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Translocation of ^{14}Carbon in Tobacco following Assimilation of ^{14}Carbon Dioxide by a Single Leaf

BY

H. JONES,[1] R. V. MARTIN, AND H. K. PORTER

(*Department of Plant Physiology, Imperial College of Science and Technology, London S.W. 7*)

With seven Figures in the Text and two Plates

ABSTRACT

$^{14}CO_2$ was assimilated by single leaves (presentation leaves) of tobacco plants for periods of 2–3 hours. The plants were then kept in air in continuous light and the redistribution of radioactivity determined at various times up to 96 hours. There was a complete turnover of sucrose in the presentation leaf in about 24 hours without change in amount. Starch turned over more slowly and simultaneously increased in amount. 20–30 per cent. of the radioactivity appeared to be irreversibly incorporated into the presentation leaf. Of the material exported from the presentation leaf some 3 per cent. reached the upper leaves and stem apex. Import into leaves above the presentation leaf was completed in about 6 hours. No activity appeared in leaves below the presentation leaf, therefore the balance of the exported activity was retained in the stem and roots.

The distribution of radioactivity in the leaves followed a well-defined pattern determined by the vascular interconnexions.

Radioautographs of stem sections provided some information concerning distribution of radioactivity in the stem.

WHEN a single leaf on a plant assimilates radioactive carbon dioxide ($^{14}CO_2$) the subsequent distribution of activity by translocation from the leaf is not uniform. For example, experiments with seedlings have shown that activity accumulates in the younger tissues, especially the apex (Aronoff, 1955). Movement to growing points has also been noted by Leonard and Crafts (1956) who applied ^{14}C-labelled 2:4-D to leaves of some woody plants and observed upward movement of radioactivity only when new growth was in progress. Crafts has also shown that in *Convolvulus* (Wild Morning Glory) young tip leaves do not export 2:4-D but mature leaves and cotyledons do. Fisher (1956) investigated the accumulation of radioactivity by *Ranunculus hirtus* leaf primordia following assimilation of $^{14}CO_2$ by one of the older leaves. He interpreted the resulting distribution of activity in the primordia as being due to selective uptake associated with a stage of development and has used this feature to determine the rate of plastochron formation. Vernon and Aronoff (1952) measured translocation rates in soybean seedlings by the rate of movement of radioactivity down the stem, and record a minimum

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value of 84 cm. per hour. They found that sucrose was preferentially translocated and that starch was the principal ethanol-insoluble component labelled. Light had no effect on the downward movement. Aronoff (1955) further showed that if only a limited area of a soybean leaf was exposed to $^{14}CO_2$ no activity appeared in the rest of the leaflet lamina, although activity moved out of the leaflet.

Porter and her co-workers (unpublished) observed that, following assimilation of $^{14}CO_2$ by a mature leaf of tobacco, radioactivity was distributed along the length of the stem but did not appear in leaves below the one treated. Distribution in leaves above the one treated suggested that the route of movement followed the vascular interconnexions. The interconnexion of leaves has been demonstrated without the aid of tracer techniques in several plants. Roach (1939) using dye-injection methods showed that leaves on the same side of the axis as the injected leaf were interconnected. He also showed that the xylem interconnexion was not only between whole leaves, but translocation of dye could occur to a restricted area of a leaf. Esau (1941) reported that when a leaf (n) of a tobacco seedling was virus inoculated, infection appeared first in leaf $n+2$ and then in leaf $n+3$. The lateral proximity of the three leaf traces at a point in the stem lower than the leaf insertions allowed phloem connexion and therefore phloem transmission of the virus.

A more extended study of the movement of ^{14}C in tobacco has now been made with the object of determining the amount and nature of the translocated material and also of providing information about the vascular interconnexions. Accordingly $^{14}CO_2$ has been supplied to single leaves of tobacco plants for limited times and the subsequent redistribution of activity determined over periods of up to 96 hours while the plants were kept in light and air. Some data on the effect of leaf age on the redistribution of assimilate has also been collected. In addition the amount of activity in ethanol soluble and ethanol insoluble fractions of the plant tissues and in starch, sucrose, and hexose was measured.

Materials and Methods

Plant material. Tobacco (var. White Burley) was grown from seed under greenhouse conditions. Plants were grown in 10-inch pots, in John Innes No. 1 compost, and given 250 mg. $NaNO_3$ and 100 mg. KH_2PO_4 per pot at about the 10th leaf stage. Under these conditions and with medium to long days the basal rosette has 8 to 10 leaves varying in length from 2 to 30 cm. The elongated axis has about 20 leaves, with a maximum leaf length of about 50 cm. at leaf 17. Axillary inflorescences appear at about leaf 30 or higher. The phyllotaxis is difficult to establish with certainty from the leaf insertions but is probably 2/5 or 3/8 in the rosette and 5/13 in the elongated axis (quoted by Allard (1942) as being the most frequent phyllotaxes in tobacco). Changing phyllotaxis in the same plant is well known.

Assimilation of $^{14}CO_2$. A single leaf attached to the plant was introduced into a perspex leaf chamber (15×26×3 cm.). The wings of the petiole were first removed and if the leaves were too large some of the basal parts and margins were removed. About 10 ml. of water was run in before sealing the chamber. 56 mg. of $^{14}CO_2$ (about 50 μc.) was circulated over the leaf using the apparatus of Porter and Martin (1952). The initial concentration of CO_2 in the system was about 2 per cent. and the whole amount was usually assimilated in 2 to 3 hours. Any residual CO_2 was absorbed in soda and its activity determined after conversion to $BaCO_3$. In some experiments small amounts of highly radioactive CO_2 were used which could be assimilated in 10 minutes. The leaves which had received treatment with $^{14}CO_2$ are called *presentation leaves*.

Illumination was either daylight or was maintained at 1,000 f.c. with H.P.M.V. lamps. Temperature at the outer surface of the leaf chamber was kept below 30° C.

At the end of each experiment leaves found to be radioactive were weighed, cut into small pieces and extracted three times with ethanol (95, 80, and 80 per cent.) and the extracts combined. Samples of the stem of about 5 g. fresh weight were similarly treated. The ethanol extracts were reduced to convenient volume and the activity determined at infinite thinness on duralumin planchettes (5·06 sq. cm.). Sugars were separated, estimated, and their activity determined as already described (Reynolds, Porter, and Martin, 1958). The residual material after ethanol extraction was finely ground in a porcelain mortar and suspended in ethanol. Aliquots of the suspension were transferred to planchettes with a pipette, dried, and the activity determined. Activity was proportional to the amount of dry material up to 1 mg. per sq. cm. In practice not more than 3 mg. of dry matter was spread on to a planchette. Some stem samples were too tough to disintegrate by hand; grinding and estimates of activity were not attempted in such cases. Starch was extracted from portions of the ethanol insoluble residue and estimated using anthrone, and the activity determined (Porter and May, 1955).

RESULTS

1. *Distribution of radioactivity and starch and sugar contents in presentation leaves receiving* $^{14}CO_2$

(*a*) *At intervals after assimilation of* $^{14}CO_2$. From each of five plants a leaf was selected which had completed its rapid-growth phase. The leaves were 27 to 30 cm. long and situated in the middle of the elongated axis. After assimilation of $^{14}CO_2$ (2 hours) each plant was kept in the light for one of the following periods: 0, 3, 6, 24, or 96 hours. One plant (0 hours) assimilated 82 per cent. of the CO_2 and the other four 100 per cent. At the end of each period the selected leaf was removed for analysis. The results are shown in Table 1 *a* and *b*. Radioautograms of the papers after chromatographic separation of the ethanol soluble extracts are reproduced in Plate 1.

Of the total activity assimilated as $^{14}CO_2$, 70 per cent. remained in the presentation leaf 3 hours after its return to air. In the next 3 hours the value

TABLE 1

Distribution of Radioactivity in Single Attached Leaves of Tobacco after Assimilation of $^{14}CO_2$

(See text, p. 495)

56 mg. $^{14}CO_2$ ($1·5 \times 10^6$ c.p.m.) circulated over the leaf for 2 hours at 1,000 f.c. or more. Plants then kept in air and light.

(*a*) Percentage of total activity supplied in the several leaf fractions.

Hours after assimilation of $^{14}CO_2$	Fresh weight (g.) of leaf tissue exposed to $^{14}CO_2$	Ethanol soluble fraction			Ethanol insoluble fraction	
		Total	Sucrose	Hexose	Total	Starch
0	—	29	23	1·4	—	—
3	6·1	28	10	4	40	19
6	7·4	15	5	2	41	20
24	7·0	11	1	1·5	28	13
96	8·0	5	trace	0·5	27	14

(*b*) Starch and sugar contents and Relative Specific Activities (R.S.A.).

Hours after assimilation of $^{14}CO_2$	Sucrose	Hexose mg./g./FW.	Starch	Sucrose	Hexose c.p.m./mg. $\times 10^{-2}$	Starch
0	—	—	—	107·6	10·4	—
3	3·8	3·6	10·7	64·9	26·7	44·9
6	3·0	2·7	10·9	32·2	13·3	38·5
24	3·9	4·1	19·2	5·2	8·1	14·9
96	4·2	4·4	22·9	0·7	2·3	11·1

fell to 56 per cent. and after 96 hours to 32 per cent. In a similar experiment the leaf was left on the plant until quite yellow and it was found that considerable activity still remained. Since the leaves were in continuous light the loss of activity must represent in the main material moved out of the presentation leaf. Two-thirds of the loss between the 3rd and the 96th hour was from the ethanol soluble fraction, which contained 30 per cent. of the supplied activity after 3 hours and after 96 hours only 5 per cent. Mainly responsible was loss of activity in sucrose (Plate 1). The ethanol insoluble fraction contained 40 per cent. of the supplied activity after 3 hours and after 96 hours 27 per cent. About half the loss was from the starch in this fraction.

The amounts of both sucrose and hexose remained unchanged so that the relative specific activity (R.S.A.) fell with time. There was thus continuous dilution of sucrose by newly assimilated $^{14}CO_2$, accompanied by continuous loss of sucrose from the leaf. The amounts of starch were apparently doubled after 96 hours in light, so that the fall in R.S.A. was here largely due to accumulation of inactive starch. The loss of total activity does suggest concomitant breakdown of starch in the light, and experiments now in progress have confirmed that this is the case.

(*b*) *In leaves of different ages.* Leaves at three developmental stages, which on reaching maturity would be in the middle of the elongated axes, were selected from three plants. The position of each leaf on a leaf-growth curve is shown in the diagram included in Fig. 4, which is based on measurements of leaf lengths. The fully expanded 6th leaf of a plant at the rosette stage was used in a separate experiment.

The three leaves of different ages were exposed to 56 mg. of CO_2 for 3 hours, after which the plants were kept in light for 6 hours and the leaves then removed for analysis. The smallest leaf assimilated 66 per cent. of the CO_2 and the other two 100 per cent. The rosette leaf was supplied with 22 mg. CO_2 (45 μc.). The results are shown in Table 2. The distribution of activity is the same in all cases as that described above. After 6 hours in light and air between 40 and 60 per cent. of the activity remained in the presentation leaves, mainly in the ethanol insoluble fraction, and less than 6 per cent. remained in the sugar. The oldest leaf retained about three times as much as the youngest, 5·4 per cent. as compared with 1·7 per cent., and activity in sugar formed a higher proportion of that in the ethanol soluble fraction in the oldest as compared with the youngest leaf. The oldest leaf also had the highest starch and hexose content.

TABLE 2

Distribution of Radioactivity in Single Attached Tobacco Leaves of Different Ages after Assimilation of $^{14}CO_2$

(See Figs. 4 and 6)

Procedure as in Table 1 except that $^{14}CO_2$ was supplied for 3 hours—followed by 6 hours in light and air.

(*a*) Percentage of total activity supplied in the several leaf fractions.

Leaf length cm.	Fresh weight (g.) of lamina assimilating CO_2	Ethanol soluble fraction			Ethanol insoluble fraction	
		Total	Sucrose	Hexose	Total	Starch
15	2·2	12·1	1·4	0·3	42·3	18·3
29	7·5	14·0	2·8	0·7	47·4	21·6
38	10·3	8·3	2·8	2·6	32·2	21·9
6th leaf of rosette	5·0	15·5	4·9	1·5	30·9	15·0

(*b*) Starch and sugar contents and Relative Specific Activities.

Leaf length cm.	Sucrose	Hexose mg./g./F.W.	Starch	Sucrose	Hexose c.p.m./mg. × 10^{-2}	Starch
15	2·4	1·2	12·5	25·1	11·3	64·4
29	2·5	1·4	8·7	23·5	10·1	50·9
38	2·5	8·3	24·1	15·9	4·4	12·8
6th leaf of rosette	1·2	0·4	4·5	111·3	99·4	91·0

2. *Movement of Radioactivity out of the Presentation Leaf*

Leaves are identified in order from the presentation leaf, *n*, that above being $n+1$ and that below $n-1$.

To examine rate of movement out of a leaf 1·4 mg. of highly active $^{14}CO_2$ was supplied to a fixed leaf area (175 sq. cm.). This amount was assimilated in 10 minutes. A leaf on the axis which had completed its growth was selected and after assimilation of the $^{14}CO_2$ the plant was kept in the light.

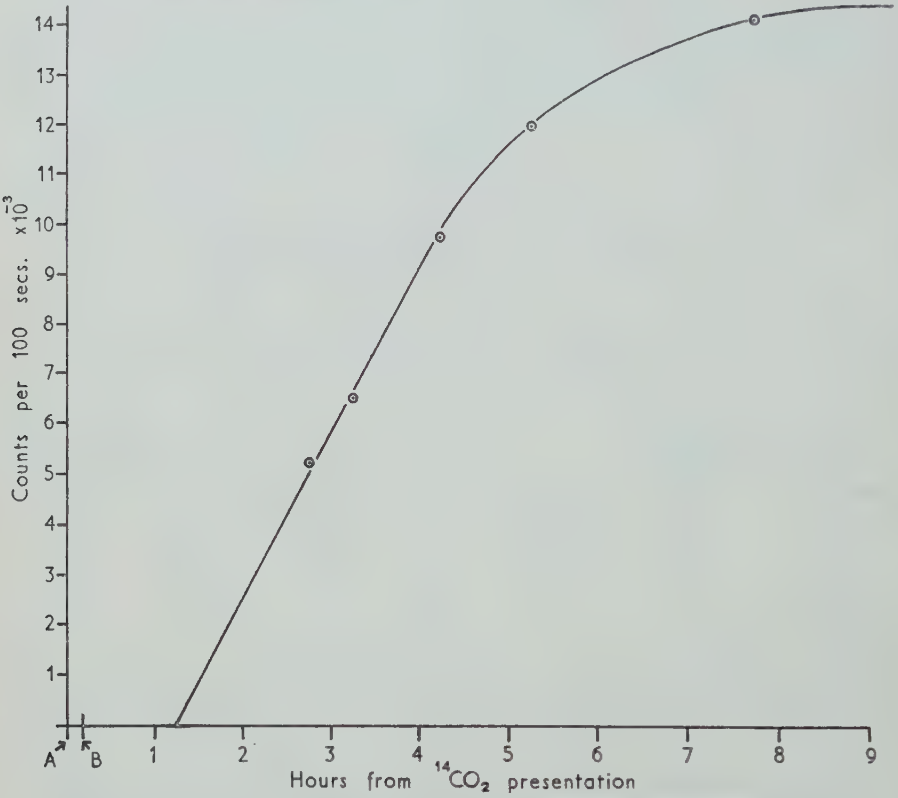

FIG. 1. Accumulation of radioactivity in leaf $n+5$ following assimilation of $^{14}CO_2$ by leaf n of a tobacco plant.

A: Start of $^{14}CO_2$ presentation.
B: Assimilation of $^{14}CO_2$ completed.

The activity in leaf $n+5$ inserted 20 cm. above leaf n was recorded. The end window of a G.M. Tube was masked leaving a small slit, and the tube placed over the same area of leaf $n+5$ at intervals up to 8 hours after the uptake of $^{14}CO_2$ by leaf n. A graph showing the increase in activity with time in one such experiment is shown in Fig. 1. Activity was first detected in leaf $n+5$ after 65 minutes. On the basis of the direct distance between the leaf insertions this means that the minimum translocation rate was 18·5 cm. per hour. Rate of accumulation of activity was constant (or nearly so) for about 4 hours after the initial entry then fell rapidly and after 7 to 8 hours very little more activity reached leaf $n+5$. As in the earlier experiments, at this stage only 40 to 50 per cent. of the total activity had moved out of the presentation leaf. Other experiments indicated that the time sequence recorded for leaf *n*+5 was the same for other leaves receiving material from leaf *n* and for the apical bud. Supply to leaves above and to the apical bud is thus restricted to the period of 6 to 7 hours following assimilation. Material lost after this time presumably moves only in the stem. Furthermore in attached leaves a proportion of assimilate is irreversibly incorporated at the site of assimilation, even when the leaf is fully expanded.

3. *General Distribution of* ^{14}C

(*a*) *In leaves and apical buds.* As already noted (p. 494) leaves below the one receiving $^{14}CO_2$ did not become radioactive, although activity moved down the stem to the roots. With one exception, discussed below, the distribution followed a constant pattern. Activity appeared in leaves *n*+3, *n*+5, *n*+8, *n*+10. These leaves were inserted on the same side of the stem as the treated leaf as illustrated in the diagram in Fig. 2. In the plant at the rosette stage activity appeared in leaves *n*+2, *n*+3, *n*+4, and *n*+5, but not in *n*+1, which was inserted on the opposite side of the axis from *n* as shown in Fig. 7. Leaf *n*+4, also opposite, had only traces of activity in comparison with *n*+2 and *n*+3.

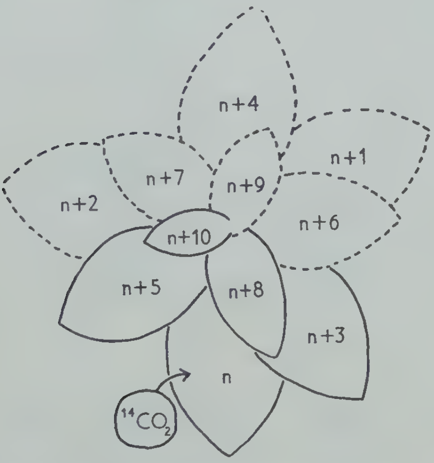

FIG. 2. Arrangement of the leaves in a mature tobacco plant which became radioactive after assimilation of $^{14}CO_2$ by leaf *n*.

Presentation leaf (*n*): 38 cm. long (see Fig. 4).
Solid outline: radioactive leaves.
Dotted outline: non-radioactive leaves.

When petioles of fully expanded leaves were injected with 0·5 per cent. acid fuchsin (Roach, 1939) dye distribution was the same as the radioactivity distribution in the leaves above the cut petiole, but dye also penetrated into leaves *n*−3 and *n*−5. Stem sections showed that dye was restricted to the xylem.

Total activity in the leaves and stem sections was determined as the sums

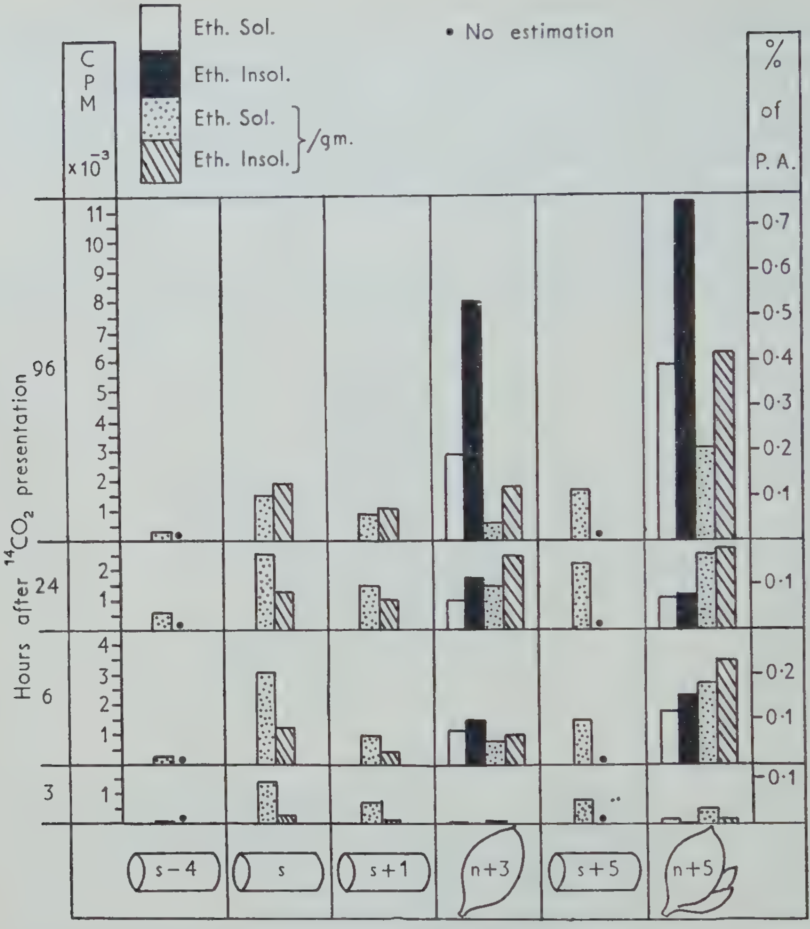

FIG. 3. Distribution of ^{14}C in stem and leaves of a tobacco plant following assimilation of $^{14}CO_2$ by a single leaf (*n*).

S: Stem section from internode below leaf *n*.
S+1: Stem section from internode above leaf *n*.
P.A.: Activity of $^{14}CO_2$ presented to leaf *n* ($1{\cdot}5 \times 10^6$ c.p.m.).
Eth. sol. and Eth. insol.: Activity in ethanol soluble and insoluble fractions.
Eth. sol./g.: Activity in ethanol soluble fraction per g. F.W.

of the activities in the ethanol soluble and insoluble fractions (see p. 495). These results are presented as histograms in Figs. 3 to 6. In the several experiments the activity in all leaves found in this way did not exceed 9·5 per cent. of that supplied in the CO_2 in plants in the rosette stage, and in more mature plants it did not exceed 3 per cent.

The increase of activity with time in leaf $n+3$ and apical bud $n+5$ and in stem pieces cut from four internodes is shown in Fig. 3 (see Table 1 for change in activity of the presentation leaf). Three hours after exposure to $^{14}CO_2$ traces of activity were found in leaf $n+3$, in the apical bud, and in the stem pieces. The amounts increase 8 to 20 fold in the next 3 hours and subsequently only slowly, so that after 96 hours the values at 6 hours were only approximately doubled. At first the amounts in the ethanol soluble and insoluble fractions were about equal but the later slow accumulation was mainly in the insoluble fraction. The highest amount recorded, in the apical bud, still only reached 0·7 per cent. of the supplied CO_2. It has already been pointed out (Table 1 *a*) that after 96 hours 70 per cent. of the activity was lost from the presentation leaf, and it is remarkable that so little appears in other leaves and the apical bud.

Similar data for movement from leaves of different ages are presented in Fig. 4 (see also Table 2). When a rapidly growing leaf received the $^{14}CO_2$ (Plant 1, Fig. 4) no radioactivity was detected in other leaves or the apical bud after 6 hours in light. The amount appearing in other leaves increased with the age of the presentation leaf and in Plant 3 was 2·5 times as great as in Plant 2 but again the total amount found was only of the order of 3 per cent. of that supplied. Simultaneous import and export in some leaves is demonstrated in Fig. 4. Leaf $n+3$ (fresh weight 8·6 g.) of Plant 3 is receiving translocate while a smaller leaf n (fresh weight 7·5 g.) of Plant 2 is exporting translocate. Distribution when the oldest leaf still green assimilated $^{14}CO_2$ is shown in Fig. 5. Activity was found in five leaves above the treated one, as well as in stem sections examined. The distinctive features were that rather little material reached the apex as compared with the two leaves below it which became radioactive, and the rather high proportion of the total activity in each leaf which was ethanol soluble. The activity appearing in other leaves was about 3 per cent. of that supplied. This plant showed an unusual distribution pattern.

Similar data for the plant at the 'rosette stage' is shown in Fig. 6. The high concentration of activity in the apical bud was noteworthy, and also the localization of the activity in leaf $n+2$ in its basal portion. The proportion of the activity supplied appearing in other leaves is the highest found in these experiments.

Measurements of activity on the leaf surface with a G.M. Tube have shown differential distribution. Some differences of this type are shown in Fig. 7. It can be seen that not only was there a gradient from tip to base, but also there was a greater concentration of activity in that half of the leaf nearer to the presentation leaf. In leaf *n* itself, immediately after the assimilation period there is a decreasing gradient from base to tip, but this is reversed after 6 hours in air, so that there is some movement within the leaf in the direction contrary to the translocation path.

(*b*) *In stems.* Of the small number of stem sections examined, maximum activity was found in that immediately below the presentation leaf. In the experiment recorded in Table 1 and Fig. 3, after 6 hours in light this stem section had more than twice the activity of the section immediately above leaf *n*. After 96 hours there was less difference between these two sites. At first most of the activity is in the ethanol soluble fraction. The relative

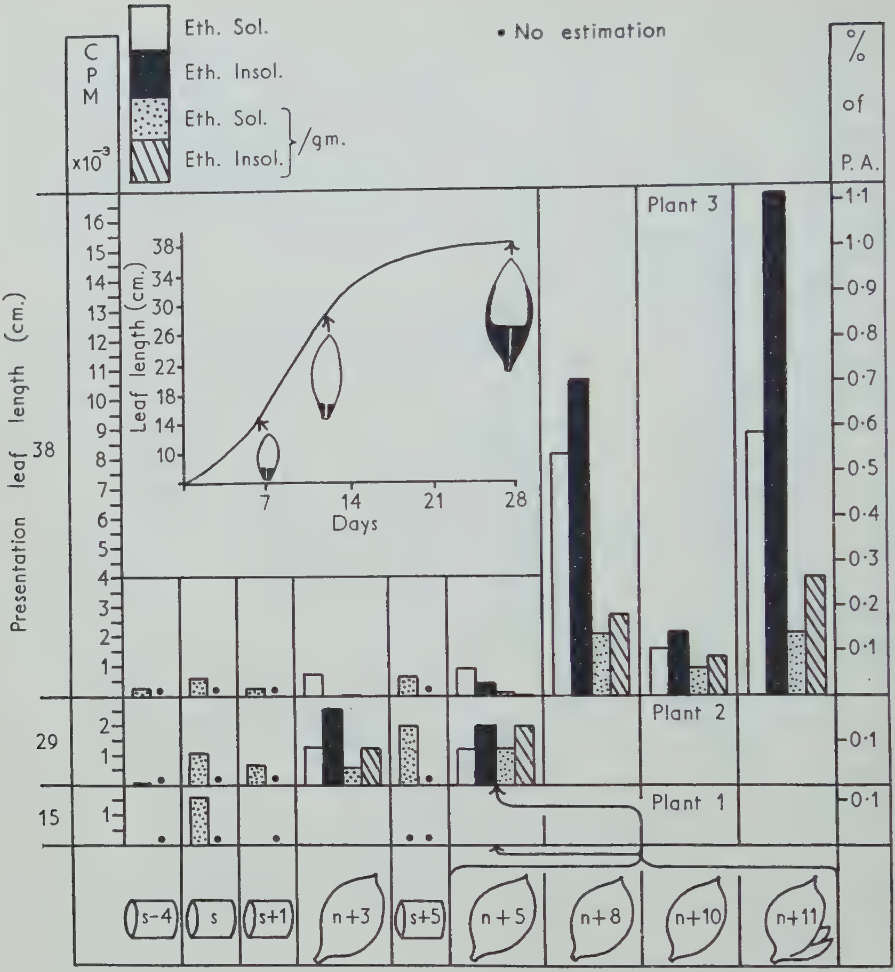

FIG. 4. Distribution of ^{14}C in stem and leaves of tobacco plants after assimilation of $^{14}CO_2$ by leaves of different ages (on separate plants).

Symbols as in Fig. 3.

Inset: Positions of the three leaves on a generalized leaf-growth curve. Black parts are sections removed before insertion into leaf chamber.

amounts of the insoluble and soluble active material slowly changes with time until the insoluble fraction predominates.

Paper chromatography of the ethanol soluble material followed by autoradiography showed that the major active component was sucrose. Finally autoradiographs of young stem sections showed marked localization of activity (Plate 2). Permanent incorporation of the ^{14}C is evident in the xylem elements, especially those recently lignified. Activity is again restricted to the side of the stem on which the presentation leaf was inserted, but even in this region inactive xylem elements are dispersed among active ones.

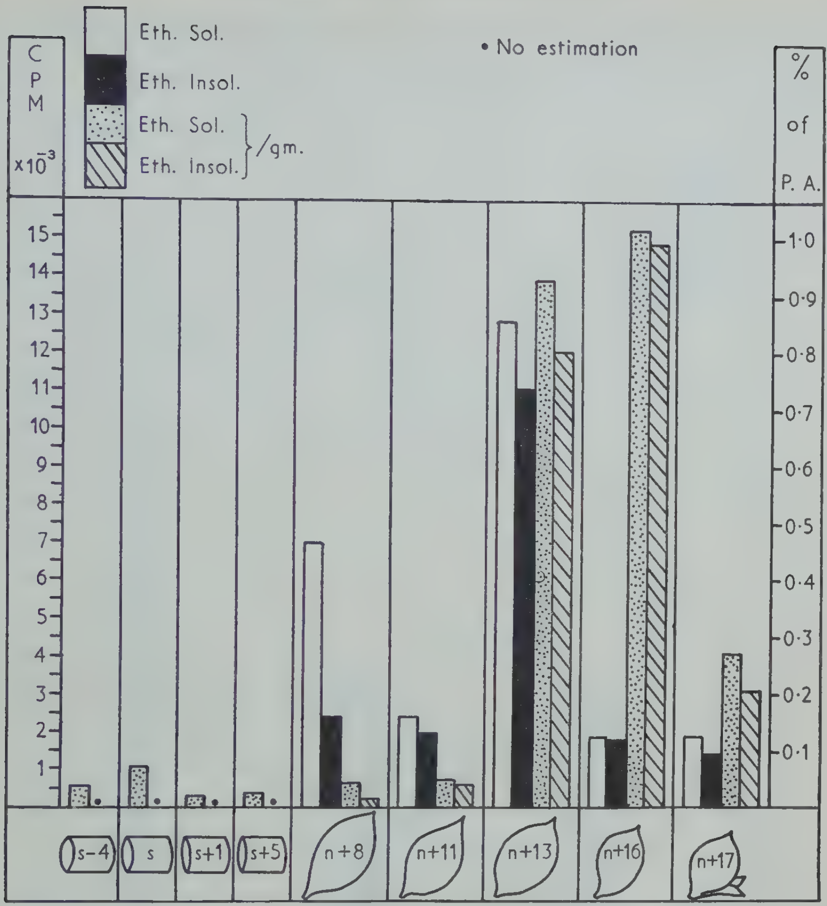

FIG. 5. Distribution of ^{14}C in stem and leaves of a tobacco plant after assimilation of $^{14}CO_2$ by the oldest green leaf.

Symbols as in Fig. 3.

DISCUSSION

A striking feature of the redistribution of radioactivity after assimilation of $^{14}CO_2$ by a single three-quarters expanded leaf of tobacco was the small proportion of the total activity which subsequently appeared in the apical bud and other leaves. About 30 per cent. of the activity was irreversibly combined in large molecules in the presentation leaf and remained when the leaf was quite yellow. About 50 per cent. was lost in a period of 6 hours following assimilation and the remaining 20 per cent. over a period of about 4 days. Since the plants were kept in continuous light the loss must represent in the main material moved out of the leaf. Upward movement proceeded at a minimum rate of 18·5 cm. per hour and was almost completed in 6 to 9 hours (Fig. 1). At most 3 per cent. appeared in the apical bud and in leaves above the site of assimilation. Quantitative measurements are not available of the amount of material moved down the stem to the roots nor of the amount incorporated into the stem. It is evident, however, that the greater part of the assimilate from a fully expanded leaf is disposed of in this way.

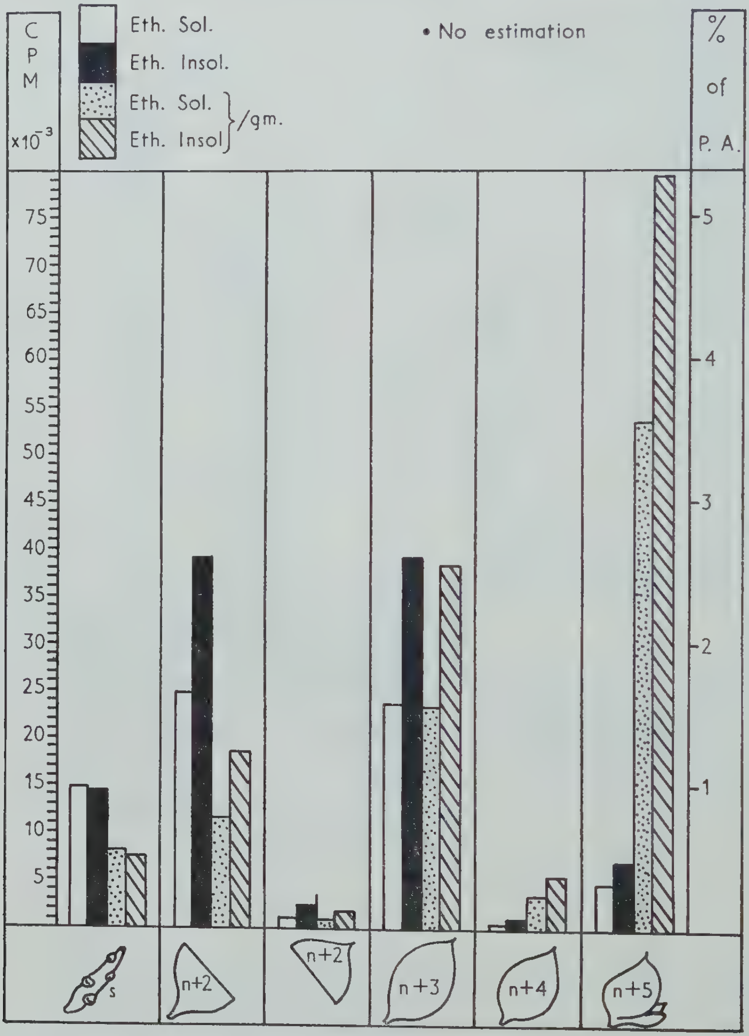

FIG. 6. Distribution of ^{14}C in stem and rosette leaves of a tobacco plant, before axis elongation.

Symbols as in Fig. 3.

Most of the activity in the apical bud was in leaf *n*+5.

Barrier and Loomis (1957) noted an initial lag in the movement of P^{32} and 2:4-D from soy bean leaves following absorption. They attributed the lag to chemical and physical transformations necessary before the labelled material could be translocated through the phloem and demonstrated (although in detached leaves) a marked 'loading' of the veins. Loading may also be due to the time required for accumulation in the transition cells or companion cells prior to discharge into the sieve tubes (Phillis and Mason, 1933). The curve in Fig. 1 also shows a lag but here assimilation was not interrupted and so no period of loading of the veins was included. The curve illustrates the gradually increasing and decreasing proportions of a constant total amount of material moving upwards which was contributed by the radioactive assimilate. The rate of translocation recorded here (see above) is considerably lower than that in some reports, for example Vernon and Aronoff (1952). This may be due to slower upward translocation in mature plants.

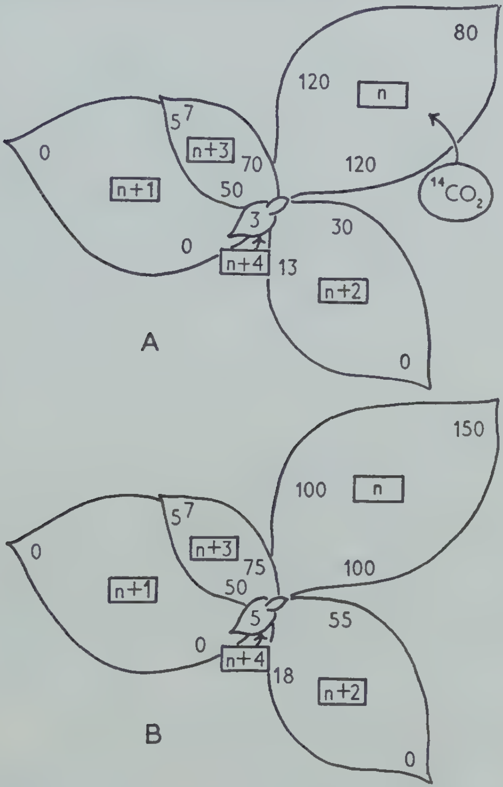

FIG. 7. Radioactivity in counts per second in seedling leaves of tobacco after assimilation of $^{14}CO_2$ by a single leaf (*n*).

A: Immediately after $^{14}CO_2$ assimilation.
B: 6 hours after $^{14}CO_2$ assimilation, plant kept in light.

Distribution of material was controlled by the vascular interconnexions, and a closer connexion was found between leaves of plants at the rosette stage than between leaves of the elongated axis, so that at the early developmental stages there was a more general distribution from a single leaf (Figs. 3–6). (See also Sharman, 1942.)

The pattern of distribution from ageing leaves after full expansion was essentially the same as at the time of nearly full expansion, but with many more leaves inserted above the presentation leaf, translocation was mainly to the uppermost leaves. When nearing the yellowing stage, sugar and starch contents tended to be somewhat higher than at younger stages, and the proportion of radioactivity in these carbohydrates was also higher.

The amount exported to other leaves was 3 per cent. of the total as compared with 0·5 per cent. from a nearly fully grown leaf. These differences are presumably associated with the declining synthesis of other leaf constituents with age. With the youngest leaf treated (Table 2) there was no movement to other leaves, so that at this stage of development carbon was retained for synthesis at the site of assimilation. Tobacco leaves do not begin to export material until about half the maximum size is reached, and there is a stage when both export and import proceed in the same leaf simultaneously. The ingoing and outgoing channels in the leaf must be separate even though adjacent in vein endings. A similar system of contiguous but functionally separate leaf supply channels exists in the stem and has been demonstrated in the present work.

Esau (1941) has recently discussed the question of bi-directional phloem movement and has pointed out that this need not necessarily occur in the same phloem strand. Sugar export, on the other hand, may occur via the phloem with transfer to the xylem taking place in the stem, in which case subsequent movement into other leaves would be in the xylem. Biddulph and Markle (1944) have demonstrated phloem export of radiophosphorus from leaves of cotton with transfer to, and upward movement in the xylem of part of the radiophosphorus. The large amount of permanent incorporation of radioactive carbon in the xylem elements in the tobacco stem at least suggests that radioactive sugars may be carried in them.

In tobacco autonomous assimilation by each leaf is the main source of carbon for its development after the leaf has unfolded. During development each leaf continuously receives small amounts of assimilate from several leaves below. As the leaf expands the amount imported declines and export begins. Exported material is dispersed through the plant in a well-defined pattern, but only a small fraction contributes to the growth of other leaves.

Secondary thickening occurs in tobacco by the time two or three leaves above the cotyledons are visible (Avery, 1933) so that vascular elements are early in complete lateral contact. It is perhaps surprising that the well-defined distribution between leaves from a single assimilation site is maintained at least over a period of days without leakage to other leaves. Owing to the secondary thickening the leaf vascular connexions cannot be observed in the older plant by the conventional histological methods. The distribution of radioactivity from a leaf n to leaves $n+3$, $n+5$, $n+8$, $n+10$, however, indicates an arrangement similar to that in the 5/13 phyllotactic system of *Linum* as described by Girolami, 1953. Leaves outside the orthostichy of the presentation leaf were found to be more radioactive in that half nearer the presentation leaf. On the above system more vascular strands would run to the nearer half and so account for the observed distribution.

The interconnected leaf system allows one leaf to supply several others and also each leaf to be supplied by several others. The organization into autonomous translocatory systems results as well in cells, though adjacent, depending for carbon on different leaves below.

In the presentation leaf the amount of sucrose remained constant but the radioactivity in sucrose declined (Table 1, Plate 1). In light therefore a steady state is maintained in which sucrose in the leaf is continuously renewed by assimilation and continuously depleted by translocation. In the present experiments sucrose was virtually completely replaced in 24 hours. Since in stem pieces 90 per cent. of the ethanol soluble activity was in sucrose the results are in accordance with the generally accepted view that sucrose is the translocatory substance and that translocation takes place mainly by day (Mason and Maskell, 1928; Goodall, 1945).

Starch increased in amount in continuous light, and it is likely that the activity decreased, although the results are not so clear cut as for sucrose. Accretion of starch in light is thus probably accompanied by a simultaneous dissolution ending in complete replacement of starch. Taken together the changes in sucrose and starch in the presentation leaf suggest that the capacity of the translocation channels to conduct material from the leaf rather than the carbohydrate supply controls movement of assimilate in light. The excess material, when a steady state is operating, is accumulated as starch.

The influence of light intensity and temperature on this steady state has yet to be investigated, but studies of the rates of sucrose and starch turnover under differing conditions might show whether they are formed simultaneously or in sequence.

ACKNOWLEDGEMENT

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EXPLANATION OF PLATES

PLATE 1

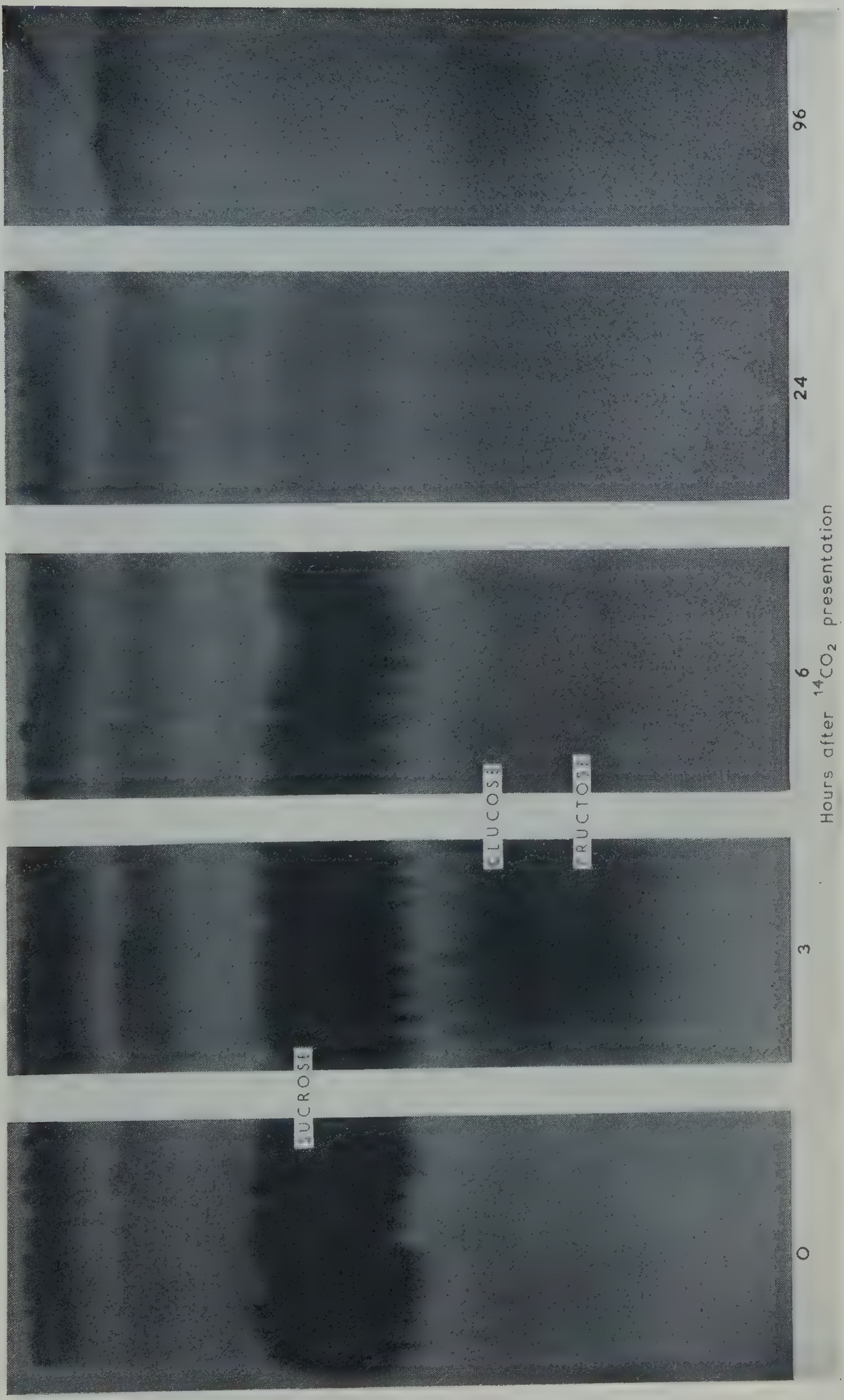
Autoradiographs of paper chromatograms of ethanol extracts of 0·4 g. F.W. of tobacco leaf tissue after assimilation of $^{14}CO_2$ by an attached leaf. Propanol/ethyl acetate/water.

PLATE 2

Details of transverse sections of actively growing stem of tobacco 60 hours after assimilation of $^{14}CO_2$ by an old leaf. Two sections 28 μ apart.

A: Stained with haematoxylin.

B: Unstained and exposed to Kodak A.R. 10 stripping film after ethanol extraction. Film subsequently developed in contact with the section.



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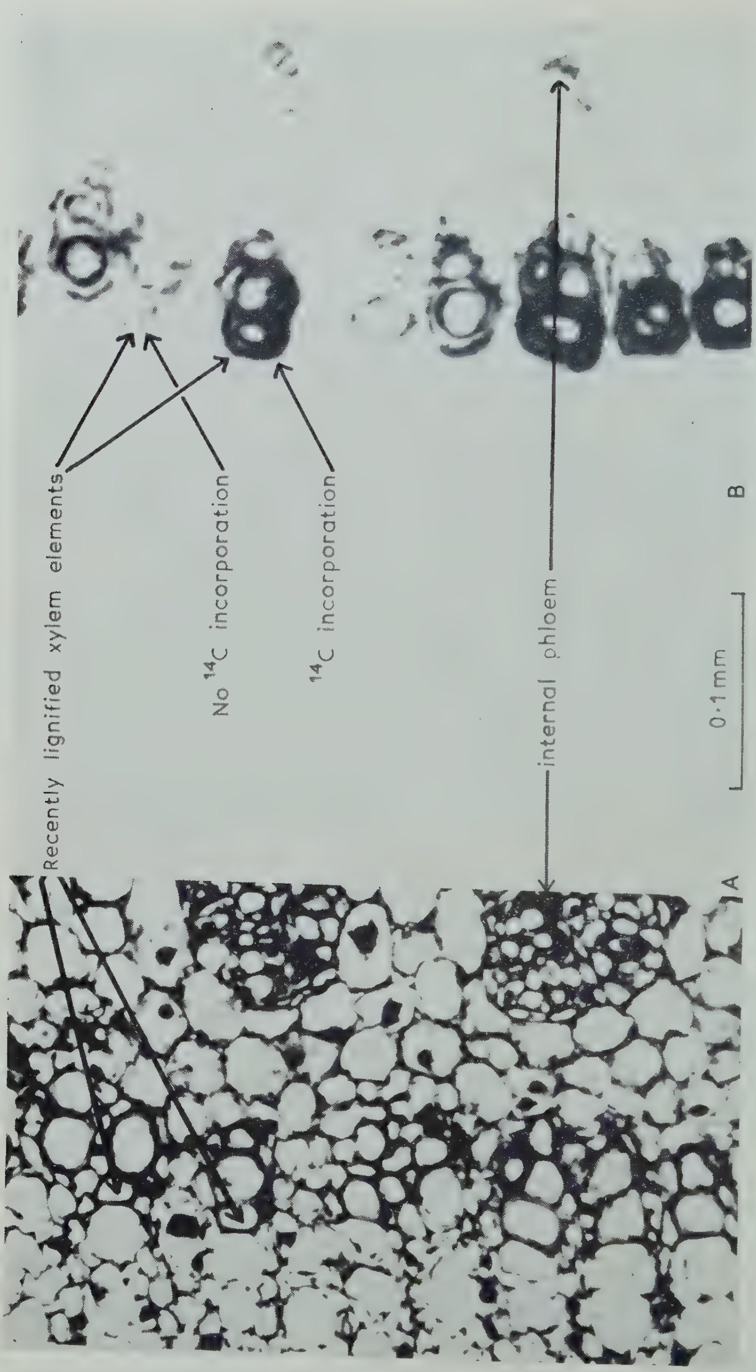

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Life-history and Cytology of *Ascocybe grovesii* Wells

BY

P. A. DIXON

(*Birkbeck College, London*)

With eight Figures in the Text and two Plates

ABSTRACT

The life cycle of *Ascocybe grovesii* Wells shows heteromorphic alternation of generations. The uninucleate vegetative hyphae, conidia and ascopores represent the haploid generation. The uninucleate ascophore hypha and its terminal cluster of asci represent the diploid. The diploid ascophore is derived from a diploid basal cell formed by the fusion of a small looping branch with an adjacent cell. The branch nucleus enters the basal cell and fuses with the nucleus already present.

INTRODUCTION

ASCOCYBE GROVESII Wells, a member of the Endomycetales, was isolated in 1951 from dressed Canadian lumber, where it occurs as a surface spoilage organism (Wells, 1954). Wells described the general vegetative, condial, and ascosporic features of the fungus and discussed the possible taxonomic position. The nature of the relationship existing between the conidial and ascosporic stages was not investigated. The conidial apparatus is of the *Cladosporium* type. The ascosporic stage consists of an erect unbranched septate ascophore bearing branched terminal clusters of asci which deliquesce to give a terminal spore drop.

In most higher fungi the diploid phase is confined to a cell (ascus or basidium initial) containing a fusion nucleus. This immediately undergoes meiosis. However, within the Endomycetales, some forms (e.g. species of *Saccharomyces* and *Hansenula*) produce a more prolonged freely reproducing phase (the diplophase), which undergoes meiosis only under suitable conditions. The existence in *Ascocybe* of the two very distinct forms of reproductive apparatus suggested the possibility of this being another form possessing a diploid phase. The work here described is an attempt to determine the relationship existing between the conidial and ascus stages.

MATERIALS AND METHODS

The strain of *Ascocybe grovesii* employed in these studies was obtained from the Commonwealth Mycological Institute (C.M.I. 1493) and maintained on 2 per cent. malt agar. All cultures used were derived from a single ascospore isolate.

The growth obtained on 2 per cent. malt agar was dense and rendered the observation of developmental details difficult. On 0·05 per cent. malt agar,

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however, although the growth on this medium was sparse, all stages were produced and the developmental details were not obscured.

It was found convenient to avoid the penetration of the agar layer by downward-growing hyphae, and the subsequent initiation of ascophores beneath the surface, by placing a layer of permeable cellophane (A. Warne & Co., London, PT 300) on the agar prior to inoculation (Robinow, 1942). Squares (2·5 cm.) of cellophane were sterilized in water by autoclaving and placed on the agar surface after draining off excess water. The inoculum, usually of spores, was streaked on the cellophane surface, the residual water assisting in the even distribution of the ascospores or conidia. All subsequent growth was confined to a single hyphal layer. Observations were made by removing the cellophane, mounting in water and covering with a cover glass.

The organism grew well on the following simple synthetic medium

KH_2PO_4	1·5 g.
$MgSO_4.7H_2O$	0·5 g.
Asparagine	0·5 g.
Glucose	10·0 g.
Fe.Zn.Mn.	1 ml. trace element solution*
Distilled water	1000 ml.

pH after sterilization 4·5

* Trace element solution prepared according to Lilly and Barnett, 1951.

This medium solidified with 2 per cent. agar was also used. The medium, being free from protein precipitate, was used in preference to malt wort for studies involving liquid culture.

Single cell isolations and controlled growth under the microscope were facilitated by using a perspex moist chamber (Dixon, 1958) and a Cook hydraulic micromanipulator (Chas. W. Cook & Co., Birmingham).

LIFE-HISTORY

The growth on 0·05 per cent. malt agar is sparse. The ascospores germinate in 12 hours at 25° C. and give rise to two or three successive germ tubes which produce a branching hyphal system. Each mycelium derived from a single spore produces a few scattered conidiophores, and mature ascophores with terminal spore drops due to the breakdown of the culsters of ripe asci.

The mature ascophore (Fig. 1 *f*) arises from an enlarged hyphal cell, the basal cell. On rich media this often bears several prop-like branches growing down into the medium. It was found that the basal cell is always united with one of the adjacent cells of the hypha by means of a small loop giving the appearance of a large clamp connexion.

A study of the development of the ascophore shows that the loop typically arises as a small branch produced just behind a cross-wall (Fig. 1 *a*). The loop which is cut off by a cross wall from its parent cell then bends over and comes into contact with the adjacent cell, the wall disappearing at the point of contact (Fig. 1 *b*). The cell with which the loop has fused then swells (Fig. 1 *c*) and from this swollen basal cell the septate ascophore is produced (Fig. 1 *d*, *c*, *f*).

The loop is usually small (Fig. 2 *b*, *c*) but variations in form occur. It can be formed from a long branch, the resultant connexion losing its clamp-like appearance (Fig. 2 *a*, *f*, *j*). Occasionally the loop can be formed from a septate branch, the looping hypha having divided before fusion (Fig. 2 *g*). The loop can also arise from a lateral branch (Fig. 2 *i*) forming a triangular connexion, or from a different hypha altogether (Fig. 2 *k*). The direction of its growth is not constant, the loop being produced on the distal or proximal side of the basal cell in relation to the growing point. This is well illustrated when two adjacent cells give rise to ascophores (Fig. 2 *h*), the loops produced being one towards and one away from the hyphal tip.

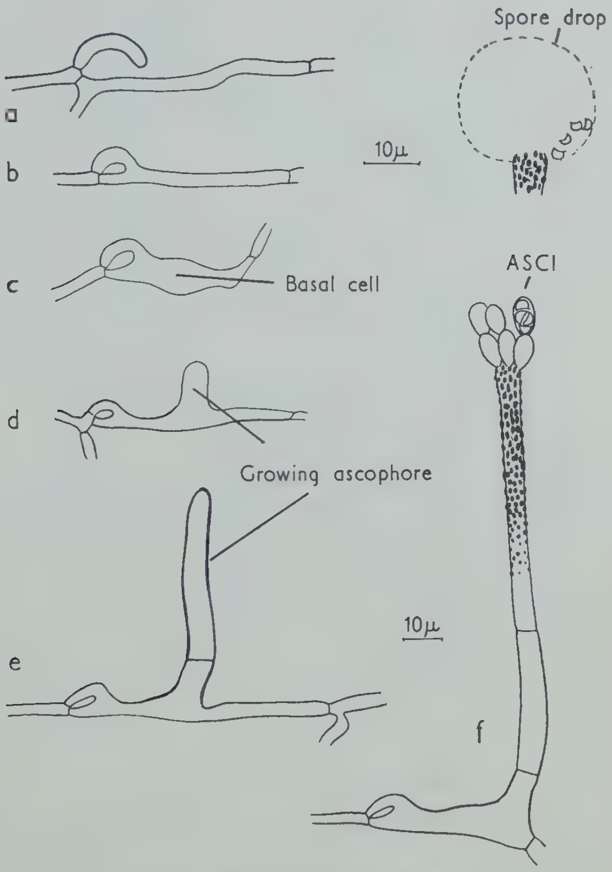

FIG. 1. Stages in ascophore formation.

A comparison with conidiophore formation shows that the loop connexion is confined to the production of ascophores. The conidiophore axis is composed of a series of elongated cells constricted at the cross-walls. The first cell of the conidiophore is budded off as a small vertical projection from a cell of the prostrate mycelium (Fig. 3 *a*). This cell is simple and no loops are involved. The second cell of the axis is budded from the apex of the first and remains attached, though the connexion is delicate. At the nodes the conidia are budded off in whorls which in turn give rise to whorls or more conidia.

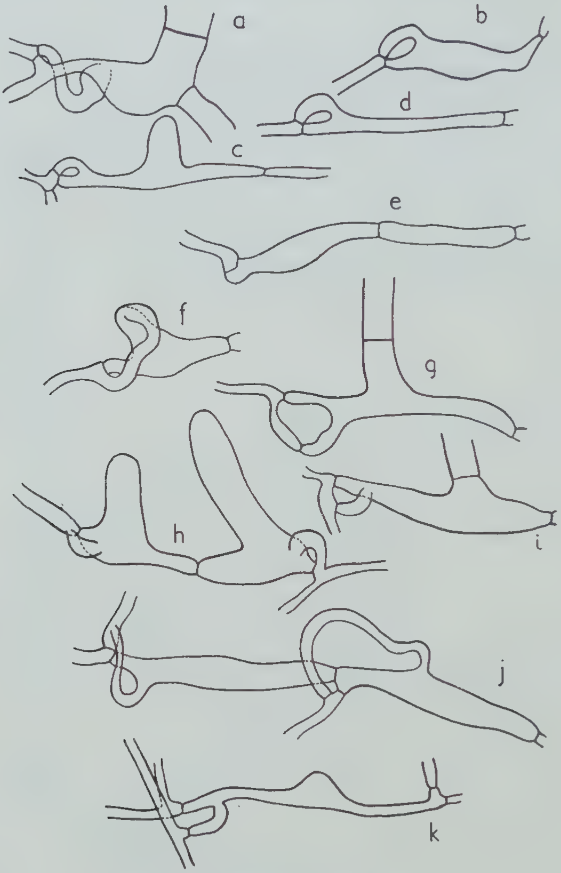

FIG. 2. Variation of loop form.

The budding process is of the narrow-base kind leaving a bud scar which stands out from the conidiophore cells as a small projection (Fig. 3 *c*, *d*).

In order to confirm that ascophore initiation is the only stage at which cell fusion takes place, the process of germination was followed. A careful study showed no variation from the simple process described by Wells.

The hat-shaped ascospore swells (Fig. 4 *b*) and produces a germ tube parallel to the flat side or brim (Fig. 4 *d*). The tube is then cut off by a cross-wall. A second germ tube is produced opposite the first (Fig. 4 *e*) and is also cut off by a cross-wall. A third germ tube is often formed opposite the brim. A noticeable feature of germination is the permanence of the brim of the ascospore (Fig. 4 *a*–*g*).

In studies on spore germination of *Ascoidea rubescens* (Walker, 1935) it

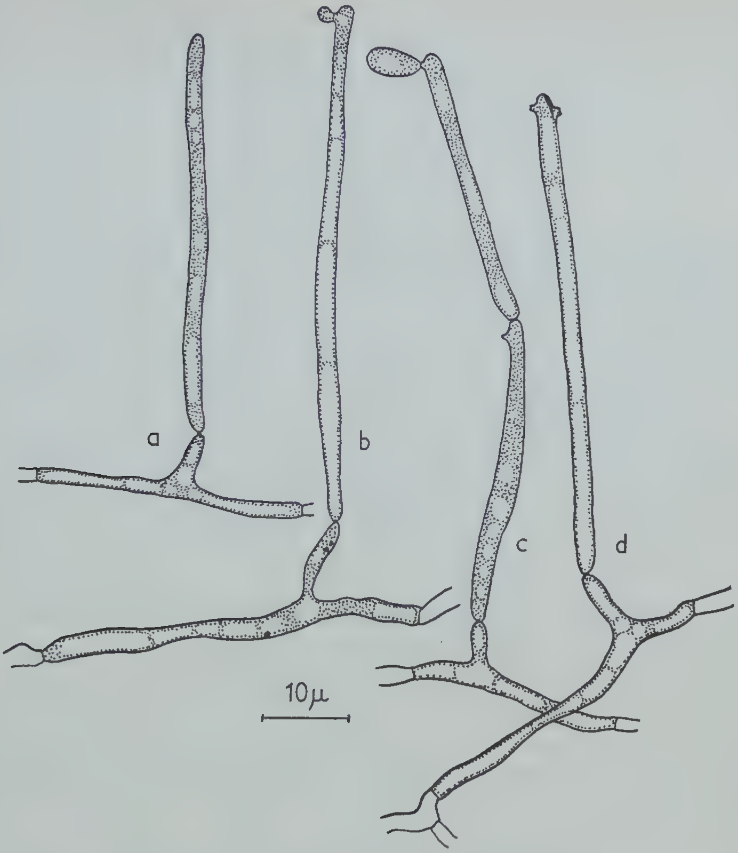

FIG. 3. Formation of conidiophore and conidia.

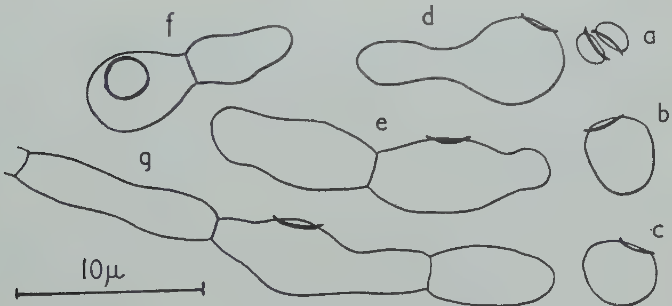

FIG. 4. Germination of ascospores in liquid culture.

was shown that two or three closely associated spores could fuse together. The resultant fusion group gave rise to a mycelium similar in all respects to that produced by a single spore. In order to test whether a similar mode of mycelium formation could be found in *Ascocybe*, ascospores were selected from a mixed ascospore suspension. These were placed in groups of two, three, or four on 2 per cent. malt agar. In all cases germination followed the normal pattern, without any cell fusions occurring.

CYTOLOGICAL STUDIES

(a) *Materials and methods*

Complete life-cycle stages were obtained using either shaken cultures or the cellophane strip technique. The staining methods employed were the same for both cultural methods, except that the cellophane cultures were processed entire and the shaken cultures in centrifuge tubes.

Staining methods. It was found that a modification of the HCl Giemsa technique (Robinow, 1942) gave the most satisfactory definition of the nuclei at all developmental stages. Fixation was carried out in Carnoys fluid, without chloroform, for a minimum period of 10 minutes, which could be extended to 12 hours without adversely affecting the quality of the fixation. Cellophane cultures were fixed *in situ* by flooding the plate with the fixative. It was found that by maintaining the fixative near freezing-point in the iceing compartment of a refrigerator, the fixation was apparently improved (Ford and Hamerton, 1956). The material was passed through a series of alcohols, from 70 per cent. to water in 10 per cent. intervals, allowing 10 minutes in each stage.

Hydrolysis was carried out using either 5N HCl at room temperature for 14–16 min. or N HCl at 60° C. for 10–12 min. The hydrolysis period required was dependent on the developmental stage being studied, the vegetative hyphae requiring less hydrolysis than the ascophore or ascus stages. Hydrolysis was stopped by transferring the material to chilled water for 5 minutes followed by a wash in Gurrs Giemsa buffer (pH 7·0). Staining was carried out for several hours in freshly prepared Gurrs Giemsa diluted 1:20 with buffer.

It was found possible to make temporary mounts using the staining solution as the mountant. The cover glasses were sealed with transparent nail varnish and the preparations retained their quality for several weeks. It was not possible to make permanent preparations using normal dehydration techniques as, under these conditions, the stain was fugitive; but by using the solid CO_2 technique (Bowen, 1956) permanent preparations were possible but not entirely satisfactory.

For some preparations aceto-orcein was used, procedure being the same as for the Giemsa technique. However, the material was mounted directly from the water wash in freshly prepared 1 per cent. orcein (Gurrs Synthetic) in 45 per cent. acetic acid.

Phase contrast. Material for phase contrast observation was obtained either from cellophane strip cultures or from shaken cultures. Direct observations were made on material mounted in water or in 15–20 per cent. gelatine (Mason and Powellson, 1956), the latter method considerably improved the contrast obtainable between the nucleus and cytoplasm.

(*b*) *Results*

The ascospores are uninucleate having a densely staining nucleus 0·8 μ in diameter (Fig. 5 *a*; Pl. 2 *k*). On germination and swelling of the spore the nucleus becomes less dense and enlarges slightly (Fig. 5 *b*). The nucleus divides during the formation of the first germ tube (Fig. 5 *c*), and the daughter nucleus passes through the isthmus joining spore and germ tube (Fig. 5 *d*). This nucleus is then cut off from the swollen spore by growth of a cross-wall (Fig. 5 *e*). The formation of the second and third germ tubes is similar leaving the swollen spore with a nucleus.

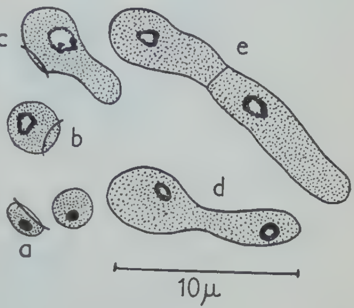

FIG. 5. Cytology of spore germination.

The mycelium produced from the spore is septate, each cell having a single nucleus. During division the separation of daughter nuclei is by a pulling out of the original nucleus into two halves (Pl. 1 *d*, *e*), passing through the dumbell shape so characteristic of previous observations on nuclear division in other members of the Endomycetales (Mundkur, 1954).

The cells of the conidiophores and conidia have a single nucleus 0·8 μ in diameter (Pl. 1 *f*). The nucleus of the hyphal cell giving rise to the conidiophore divides within the short vertical upgrowth of the conidiophore primordium and the daughter nucleus enters the first conidiophore cell, the parent nucleus remaining in the projection of the basal cell. The nucleus of each conidiophore cell is situated apically near the site of the spore formation and divides when the budding conidium is about two-thirds of its mature size, the daughter nucleus squeezing through the narrow connecting stalk between conidiophore and conidium.

The morphological and cytological processes during ascophore development appear to be as follows. The initiation of the ascophore basal cell is the result of the fusion of the loop with an adjacent uninucleate hyphal cell. The loop is formed in exactly the same manner as a branch, the hyphal nucleus divides, the daughter nucleus passes into the short branch (Pl. 1 *j*) and a cross-wall is laid down at the base of the branch. The loop comes into contact with the adjacent cell, and fuses. The separating wall breaks down and the loop nucleus passes into the uninucleate cell giving a binucleate cell (Fig. 6 *a*; Pl. 1 *l*). The two nuclei fuse giving a large single fusion nucleus (Pl. 1 *a*, *k*). The fusion or basal cell swells and produces the upward growing ascophore initial (Fig. 6 *b*, *c*, *d*, *e*; Pl. 1 *g*, *h*, *i*) which gives rise to the erect septate ascophore of uninucleate cells. During its growth the older cells become highly vacuolate with each nucleus half-way up the cell against the wall.

The uninucleate condition of the ascophore cells is clearly demonstrated by phase contrast microscopy (Pl. 1 *b*, *c*). The nuclei are visible as small dark spheres surrounded by a clear halo of varying size, the nucleus of the terminal cell being particularly distinct. The picture obtained using either orcein or Giemsa stains differs in that the nucleus appears as a large densely staining body often 3–4 μ in diameter (Pl. 1*i*).

The length of the ascophore depends on the concentration of the medium. On 0·05 per cent. malt agar it is composed of only 3 or 4 cells, but on 2 per cent. malt agar it may be much larger consisting of 10–12 cells. The incipient asci of the basal whorl are formed in succession. From the apex of the ascophore bud cells (potential asci) are formed in succession, the first cell being an exactly terminal bud. The second and succeeding buds are produced around this central cell, although the order of their production is difficult to determine. Each basal cell buds again terminally and a whorl of cells is again produced around this central cell. The order of budding is often synchronous. The first-formed basal cell may form its first bud whilst the ascophore apex is producing the second cell of the basal series (Fig. 7 *a*).

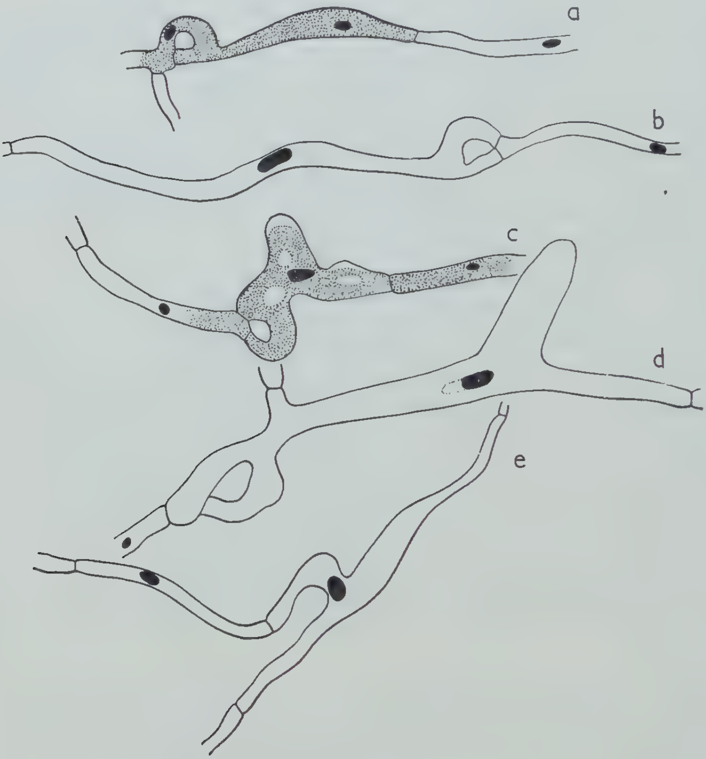

Fig. 6. Cytology of ascophore basal cell.

During the formation of a basal cell the nucleus of the ascophore terminal cell divides when the daughter cell is quite large (Fig. 7 *d*) and appears to pull apart into daughter and parent nuclei. Similar dumbell figures occur when the basal cells give rise to daughter cells. With the techniques employed no division figures other than this type were seen.

Conversion of bud cells into asci usually takes place in basipetal succession. The oldest central whorl of buds will begin to form spores even when the peripheral regions of the head are still budding. The nucleus of the ascus, 1·0–1·4μ in diameter, divides into two, but unlike the nuclear divisions during ascus formation, some details can be seen. The nucleus becomes less dense, granular, and with a suggestion of threads (Fig. 7*o*; Pl. 2*b*). This stage is succeeded by the nucleus becoming lobed, six lobes often being visible (Fig. 7*l*, *n*). The nucleus finally separates (Fig. 7*h*) into two halves. The two nuclei either remain in the centre of the cell (Pl. 2*c*; Fig. 7*k*) or migrate to the poles of the cell (Fig. 7*i*). These two nuclei then divide again to give the four nuclei of the ascospores (Pl. 2*d*, *e*). The orientation of the plane of division is not constant, the four nuclei being either formed in the central region of the cell (Fig. 7*k*, *j*), or as two pairs at opposite ends of the cell (Fig. 7*j*, *k*, *m*). The final position of the spores (Fig. 7*i*, *p*; Pl. 2*f*, *g*, *h*)

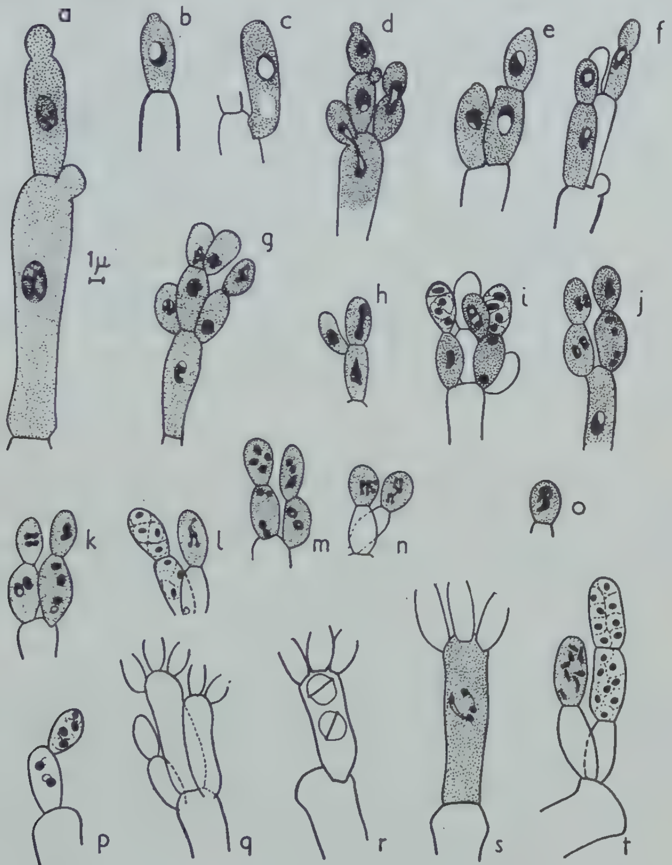

FIG. 7. Cytology of ascospore formation.

is determined by their cucculate form, rather than by the position taken up by the nuclei at their final division. Ascospore formation proceeds downwards through the whorls of asci to the basal whorl (Fig. 7*r*, *s*; Pl. 2*j*, *k*). In the large basal cells the spores are much larger (Fig. 7*r*). The spores are released by break down of the ascus wall. Only the tip of the ascus dissolves (Fig. 7*q*, *r*, *s*) leaving the remains of the ascus wall as an elongated cup attached to the supporting cell beneath.

Occasionally eight-spored asci are found. These are formed, not as single aberrant cells in a cluster of four-spored asci, but as an entire terminal cluster wherein each ascus contains eight spores (Pl. 2*l*). The formation of asci with more than the normal number of spores has been investigated in *Saccharomyces* and shown to be due to a rare extra mitotic division of one or more spore nuclei. The possibility of this event taking place in all the cells of a cluster would seem unlikely unless a genetic mutation has taken place during ascophore formation. It is more likely that the ascophore has given rise to tertaploid asci, a situation comparable to that in *Aspergillus nidulans* (Pontecorvo, *et al.*, 1953) where diploid strains give rise to perithecia containing sixteen-spored asci as opposed to the normal eight.

DISCUSSION

The life cycle of *Ascocybe grovesii*, illustrated in diagrammatical form in Fig. 8, can be interpreted as showing an alternation of generations. The haploid generation is confined to the ascospores, vegetative hyphae and the conidial apparatus, and the diploid to the ascophore and its terminal cluster of asci.

Within the fungi the only examples hitherto described of alternation of generation tend to be isomorphic. In *Allomyces* the two phases are morphologically similar but bear different types of sporangia (Emerson, 1941). In *Saccharomyces* and other yeasts the cells of the diplophase are similar to those of the haplophase except that they tend to be slightly larger. This is a reflection of the increase in ploidy, since a series of haploid, diploid, triploid, and tetraploid strains show increase in size as the ploidy increases (Lindegren, 1949). In *Ascocybe* the ascophore is in structure quite distinct from the vegetative hyphae and conidial apparatus and the life cycle can be considered a heteromorphic one.

In contrast to the alternation found in *Allomyces* and *Saccharomyces*, where the diplophase and haplophase are separate, the two phases in *Ascocybe* are normally intimately connected. The ascophore depends upon the prostrate hyphal system for the translocation of nutrients from the growth medium and this is in many respects similar to the situation found in mosses.

Attempts have been made to study the growth of isolated ascophores. These structures separated by micromanipulation from the vegetative hyphal system, and placed on solid nutrient medium, have shown some growth. The cells of the ascophore produce one or two stout branches, similar to the parent cell. These branches immediately grow away at right angles from the surface of the medium and produce whorls of asci at their apices. It is possible, with a microneedle, to lay the erect tips of the branches down on to the surface of the medium. Growth continues and the tip immediately grows away again from the surface. This is apparently a thigmotropic response. Further work to eliminate other possible explanations is in progress. These results indicate that the diploid phase is capable of independent growth, though whether this is due to the use of nutrient material already present in the ascophore cells prior to separation or to the direct absorption of materials from the medium is not certain. It also shows that the ascophore normally does not become independent since any growth is directed towards the production of aerial clusters of asci.

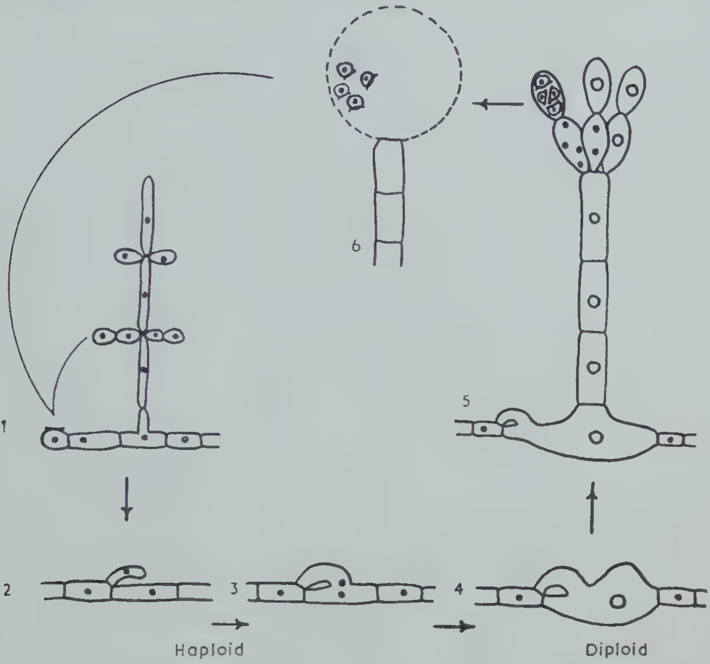

FIG. 8. Diagrammatic life cycle of *Ascocybe grovesii* Wells.

In shaken liquid culture it is possible, by serial transfer, to obtain pellets up to one centimetre in diameter. These are predominantly composed of ascophore hyphae which are extremely long and freely branched and it appears probable that the size of the ascophore on solid media is only limited by the amount of available nutrient translocated by the basal hyphal system.

SUMMARY

1. The life cycle of *Ascocybe grovesii* Wells shows an alternation of generations. The vegetative hyphae, conidia, and ascospores are haploid. The ascophore and terminal cluster of asci are diploid.

2. The ascophore arises from a diploid basal cell. This is formed by the fusion of a small looping branch with an adjacent cell, this fusion cell becoming the basal cell.

3. The conidia, hyphae, and ascospores are uninucleate. The fusion cell is initially binucleate due to the migration of the branch nucleus. The two nuclei fuse to give a large diploid fusion nucleus.

4. The cells of the ascophore are uninucleate. The terminal cell buds to give a terminal cluster of diploid asci.

5. Meiosis occurs in the cluster in basipetal succession. No regularity of nuclear position is detectable in the ascus during meiosis, the final position being determined by the shape of the spores.

6. Normally four-spored asci are produced but some terminal clusters are composed entirely of eight-spored asci.

Acknowledgement

I would like to thank Professor C. T. Ingold for his advice and encouragement during the course of this work.

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EXPLANATION OF PLATES 1 AND 2

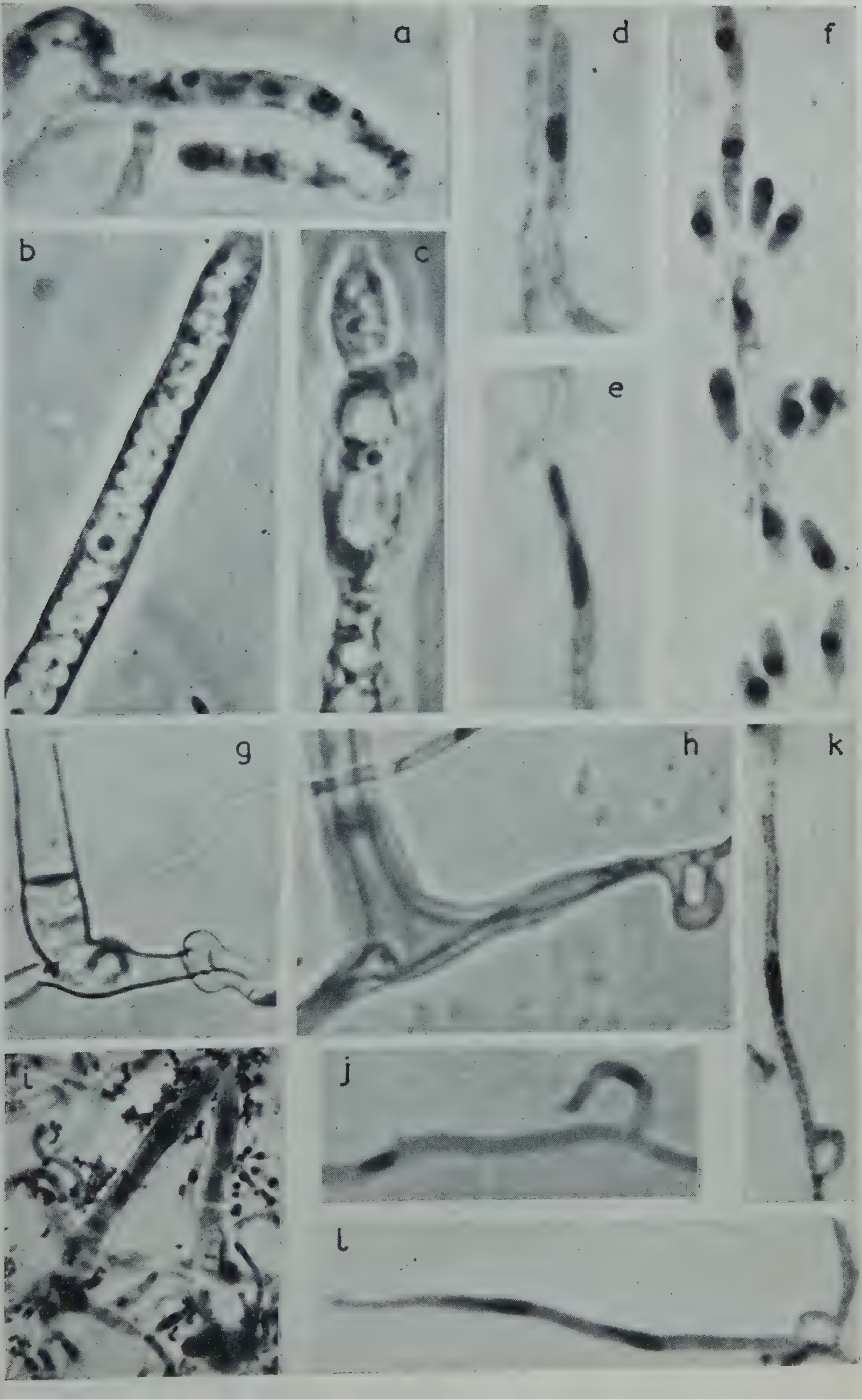
Plate 1

a, b, c are Phase contrast, and *d, e, f, i, j, k, l* Giemsa preparations.

a. Fusion cell. × 1,700.
b. Terminal cell of ascophore. × 1,500.
c. Apical buds of ascophore. × 1,500.
d, e. Mycelial nuclei. × 2,000.
f. Conidiophore and conidia. × 2,000.
g, h. Loop at base of ascophore. × 1,200.
i. Large nuclei in ascophore cells. × 1,000.
j. Loop nucleus. × 1,500.
k. Single fusion nucleus. × 1,000.
l. Two nuclei in fusion cell. × 1,000.

Plate 2

a–h. Stages in the formation of ascospores. × 3,000.
i. Spore drop. × 2,000.
j, k. Divisions in three columns of asci at different stages of maturity. × 3,000.
l. Eight-spored asci. × 2,000.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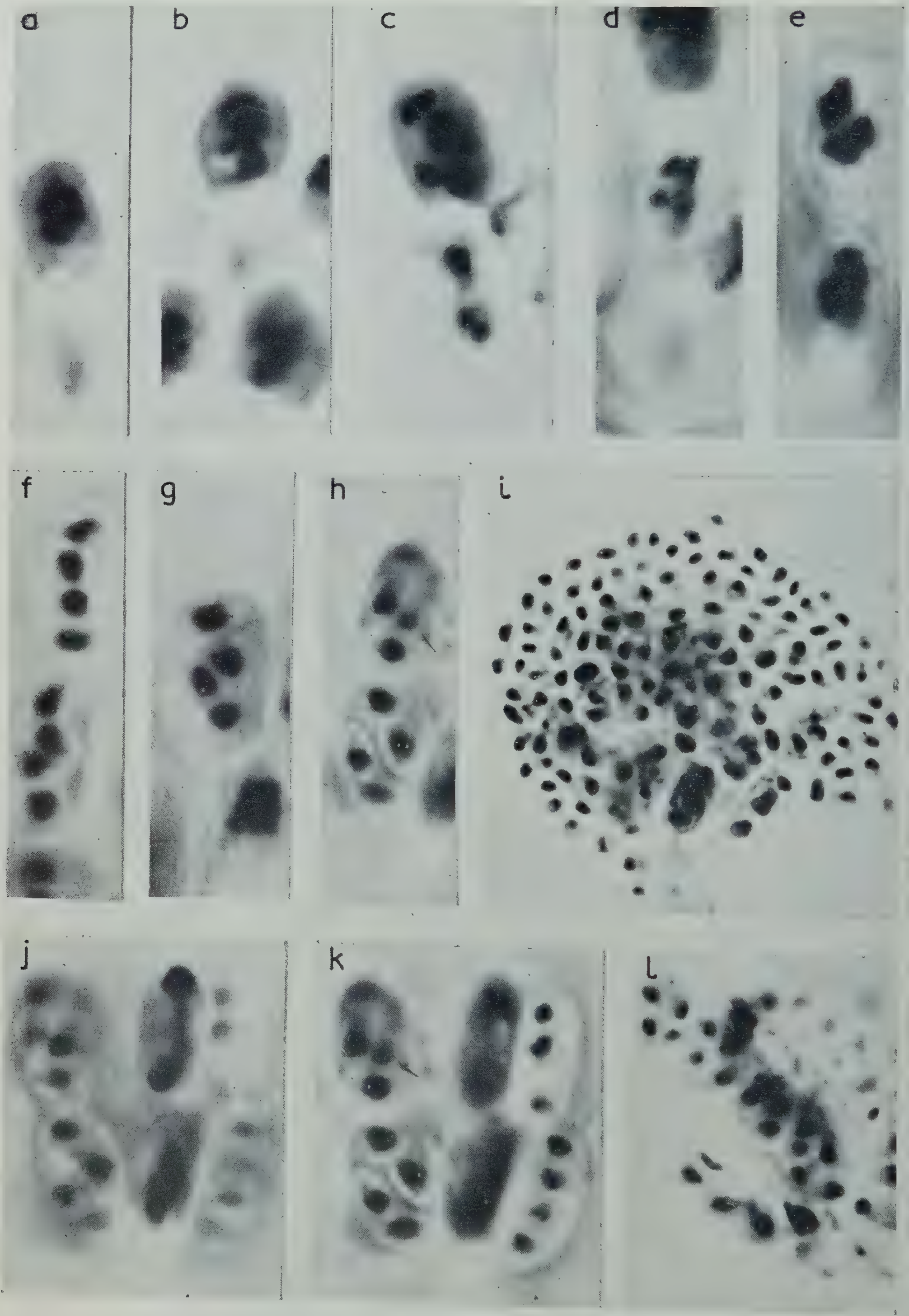

P. A. DIXON

Environmental Control of Flowering in *Vicia faba* L.

BY

L. T. EVANS[1]

(*Earhart Laboratory, California Institute of Technology*)

With seventeen Figures in the Text

ABSTRACT

There is a heteroblastic change in leaflet number in many stocks of *Vicia faba*, the rate of change being affected by the temperature and photoperiod under which the plants are grown. In all except the earliest flowering stocks of broad beans, and particularly at high temperatures, flower initiation shows a quantitative long-day response. For full development of the initiated inflorescences long days are required.

Flower initiation may be accelerated in all except the earliest flowering stocks of *V. faba* by brief exposures to low temperatures, particularly when the plants are grown in short days at high temperatures. The response to low temperatures is more rapid at 10° C. than at 4° C. but eventually approaches saturation at both temperatures. More prolonged exposure to low temperatures delays flower initiation. The response to low temperatures increases with increasing plant age but can occur during embryo development on the mother plant.

At temperatures above 14° C., and particularly above 23° C., a reaction inhibitory to flower initiation occurs. This reaction is probably restricted to the diurnal dark periods but is operative at all stages of the life cycle, including embryo development. Its inhibitory effects may be overcome by subsequent cold treatment, and when the low temperature processes have reached saturation subsequent high temperatures are no longer inhibitory.

Although nucleosides could accelerate flower initiation, purine and pyrimidene analogues did not, with one exception, reduce the response to low temperature treatment.

INTRODUCTION

THE effects of environmental conditions on the growth and development of *Vicia faba* have not been investigated extensively. That some stocks of broad beans may initiate flowers in complete darkness has been shown by Goebel (1900), Grainger (1948), and Chakravarty *et al.* (1956). David (1946) states that some stocks of *V. faba* are short-day plants, while Ramaley (1934) found the species unaffected by day-length. In their investigation of the photoperiodic response of crop plants of various geographical origins, Doroshenko and Rasumov (1929) found that whereas day-length had no effect on the earliness of flowering in *V. faba major* from Italy, day-lengths of 9 and 12 hours caused a marked delay in the flowering of *V. faba minor* from Turkestan, and a slight delay in strains from several altitudes in Abyssinia, while Warington (1933) and Robitzsch (1938) also found that short days retarded

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and reduced flowering and seed setting. Both Noye (1945) and Chakravarty *et al.* (1956) have observed that whereas flower initiation could take place in short days, full flower development was favoured by long days.

The responses of *V. faba* stocks to vernalization appear to be equally diverse. Robitzsch (1938), with a range of early and late-flowering stocks, and Souza da Camarra (1934), with *V. faba equina*, found no vernalization response, while Muratova (1931), Tanaka (1936), Ballatore and Mirto (1956), and Chakravarty *et al.* (1956), hastened flowering considerably by cold treatment of a range of *V. faba* stocks.

It appears, then, that although most stocks of *V. faba* demand neither vernalization nor long days for flower initiation, many, particularly the winter field beans, may respond to both conditions. Such quantitative responses may yield information which is not obtainable from plants with qualitative responses. In the following pages the effects of a wide range of controlled environmental conditions on flowering in several stocks of *V. faba* are described.

MATERIALS AND METHODS

The *V. faba* seed stocks used were, with one exception, obtained from the Director of the National Institute of Agricultural Botany at Cambridge. They are listed below, in the order of increasing lateness of flowering.

Dutch Weir spring beans (BEN 226)
Suffolk Red spring beans (FB 161)
Ferry Morse Dwarf broad improved longpod
French winter bean (FB 158)
Bullocks winter bean (BEN 281)
Gartons P/L14 Giant winter bean (BEN 270)

Nearly all proved to be rather variable in their flowering behaviour, especially under conditions adverse to early flower initiation, as may be seen from the estimates of standard error included in the succeeding figures.

The experiments were carried out in the Earhart Laboratory at Pasadena, the organization of which has been described by Went (1957). All plants were grown singly in small pots of mixed gravel and vermiculite, which were saturated with nutrient solution once each day and given water as required to prevent wilting. Root nodules were absent from all plants. Except in the pilot experiments, all lateral shoots were removed as they became apparent.

Time of floret appearance was recorded when the microscopic inflorescences first became visible in the twice-weekly inspections of all plants, and the node of first flowering was recorded later, taking the cotyledonary node as O.

HETEROBLASTIC DEVELOPMENT OF LEAVES

The first and second leaves of the plumule of *V. faba* are reduced to small scales and the succeeding leaves, which are borne alternately in two rows, consist of two or more leaflets. The growth and shape of the leaflets has been analysed by Sirks (1929), who found an increase in the mature size of the basal

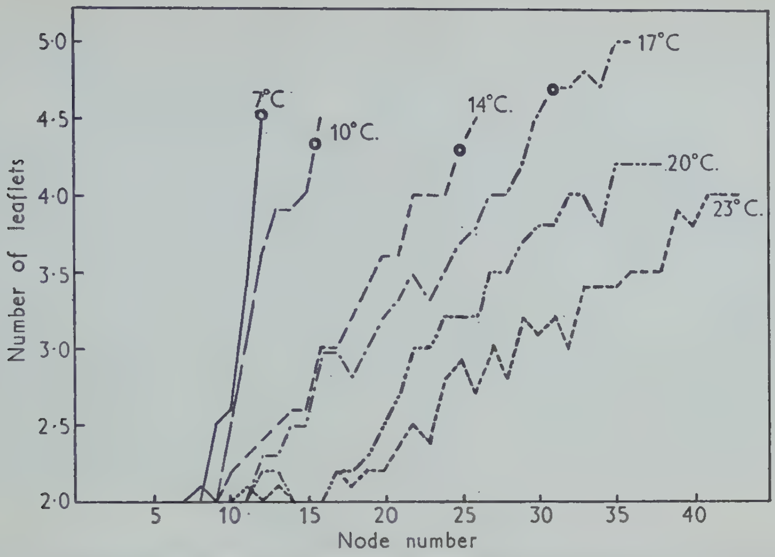

FIG. 1. The effect of temperature on the rates of increase in leaflet number with node number for Bullocks winter bean plants grown in 8-hour photoperiods. o indicates the mean node of first flowering.

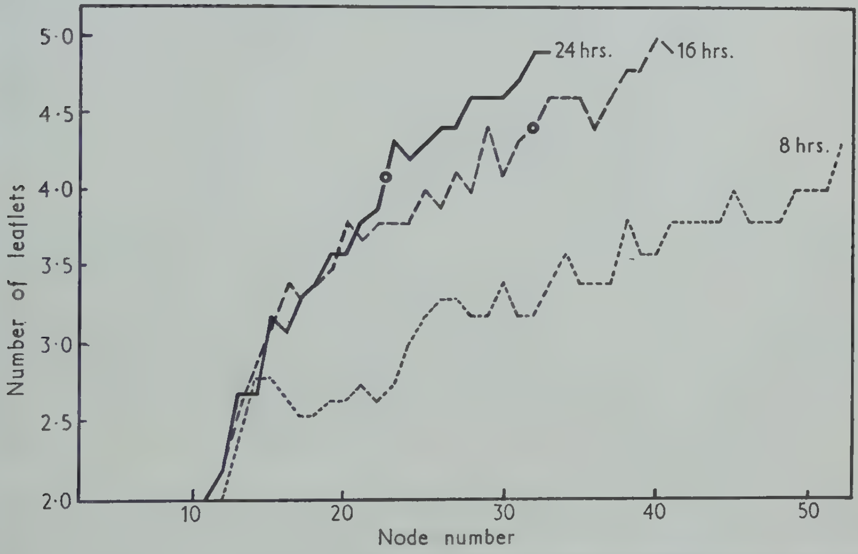

FIG. 2. The effect of photoperiod length on the rates of increase in leaflet number with node number for Bullocks winter bean plants grown at 23°–17° C. o indicates the mean node of first flowering.

leaflets up to the 6th node. A similar result was obtained with plants used in the present study, the mature size tending to be greater the longer the photoperiod and the lower the temperature of growth. Beyond the 6th node the size of the basal leaflet remains relatively constant under given conditions, but there is a tendency for the number of leaflets to increase. In Figs. 1 and 2 the increases in leaflet number with node number are plotted for groups of 8–10 plants of Bullocks winter beans grown under various conditions of temperature and photoperiod. It may be seen that the rate of increase in leaflet number rises sharply as the temperature of growth falls, and, to a lesser extent, as the photoperiod increases. The most striking feature of these examples of heteroblastic development was the constancy with which flower primordia first developed at either the last node in each row with four leaflets or the first with five, even when one row lagged behind the other. Over a very wide range of conditions the mean leaflet number at the node of first flowering only varied from 4·2 to 4·7, and by extrapolating from the results of early leaflet number counts it was possible to estimate in advance the approximate node of first flower initiation. Other stocks of *V. faba*, especially very early flowering ones such as Dutch Weir and Suffolk Red spring beans, did not show such pronounced effects of temperature and photoperiod, nor did they have such a high mean leaflet number at the first nodes of flower initiation.

RESPONSE TO PHOTOPERIOD

Figs. 3 and 4 indicate the effect of photoperiod on the time to the appearance of flower initials and on the node of first flowering in unvernalized plants of four stocks of *V. faba*, ranging from the very early Dutch Weir spring beans to the late flowering Bullocks winter beans. There were 8 to 12 plants in each treatment and all were grown from germination in the photoperiods indicated. They all received 8 hours of natural daylight each day in an ambient temperature of 23° C., after which all were moved to a temperature of 17° C. for the dark period or for supplementary illumination with light of 500–700 foot candles intensity from fluorescent and incandescent sources.

All four stocks show a quantitative long-day response. Even the earliest flowering Dutch Weir spring beans show a significant reduction in time to flowering with increase in the photoperiod from 8 to 12 hours. There is a slight, but not significant, tendency for the response to lengthening of the photoperiod to increase with lateness of flowering. At other temperatures the relation between time to initiation and length of photoperiod is not always linear. The latest flowering stock used, Garton's giant winter beans showed no flower initiations in either 8- or 16-hour photoperiods at 23°–17° C. unless previously vernalized.

In no stock was the node of first flowering reduced to the same extent as the number of days to floret appearance by increase in the photoperiod, while in the earliest flowering Dutch Weir spring beans the mean node of initiation was unaffected by the photoperiod.

In Fig. 5 the results already quoted for days to floret appearance and node of first flowering in French winter beans are given along with data for the rates of leaf appearance and increase in length of the main shoot, as well as the estimates of the time to flower initiation obtained from application of the node of first flowering results to a set of lines depicting node formation under the various photoperiodic conditions. The close agreement between the

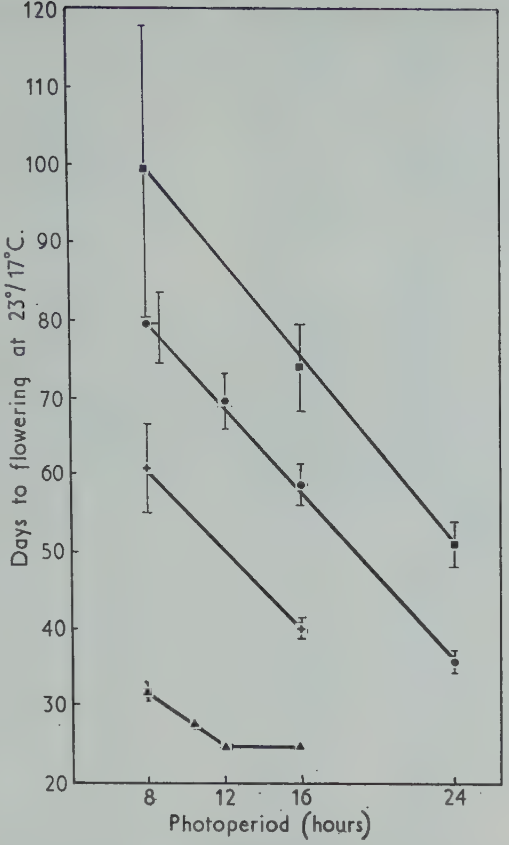

FIG. 3

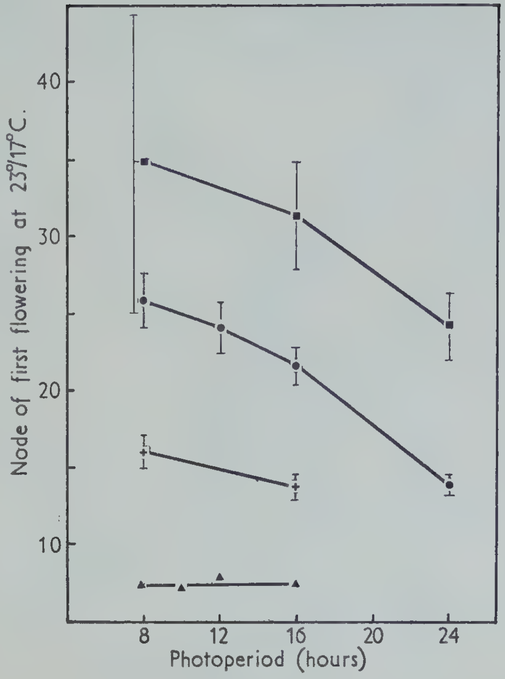

FIG. 4

FIGS. 3 and 4. The effect of photoperiod length on the time (Fig. 3) and node (Fig. 4) of first flowering in four stocks of *Vicia faba*: Dutch Weir spring beans (▲), Suffolk Red spring beans (+), French winter beans (●), Bullocks winter beans (■).

results for the rate of stem growth and the rate of node formation suggest that growth responses to day length are due entirely to changes in the rate of node formation rather than to changes in internode length. In fact, the rate of node formation increases with lengthening of the photoperiod almost as much as the rate of progress to flower initiation, which accounts for the much smaller effect of an increase in day-length on node of first flowering compared with that on the time to initiation or to floret appearance.

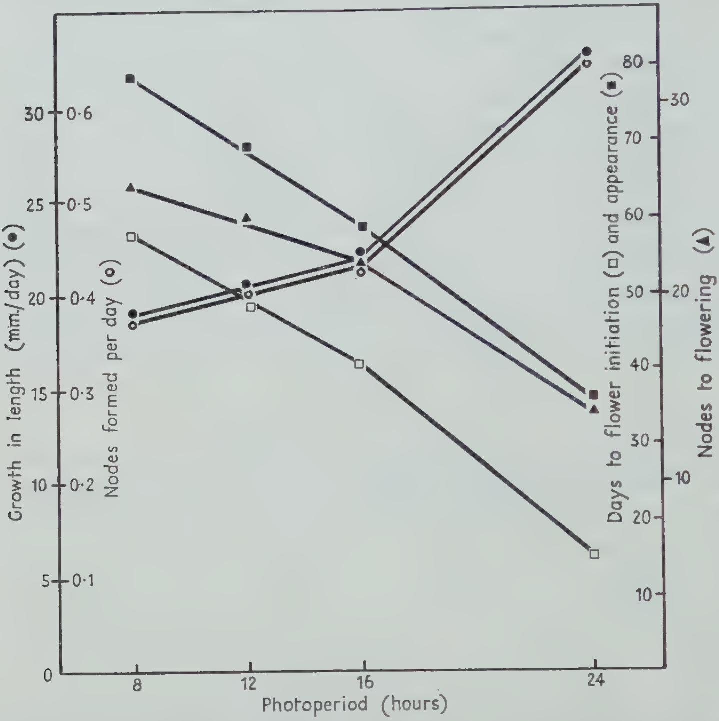

FIG. 5. The effect of photoperiod length on French winter beans grown at 23°–17° C. The vertical scales, from left to right, are for: growth in length in mm. per day (●); number of nodes formed per day (o); days to flower initiation (□) and floret appearance (■); number of nodes to first flowering (▲).

EFFECTS OF TEMPERATURE

On the day-length response. Figs. 6 and 7 indicate the times to floret appearance and nodes of first flower initiation in French winter beans grown from germination in three day-lengths at various temperatures. There were 8–10 plants in each treatment, and they were grown in natural daylight for 8 hours each day, at temperatures of 30°, 26°, 23°, 20°, and 17° C., and then transferred to darkness or supplementary artificial light in temperatures 6°–7° C. lower. The 23°–17° C. treatments were sown in December 1954, and those in 20°–14° C. were sown a few weeks later, while the remainder had to be sown still later, in May 1955, and thus developed during months of higher

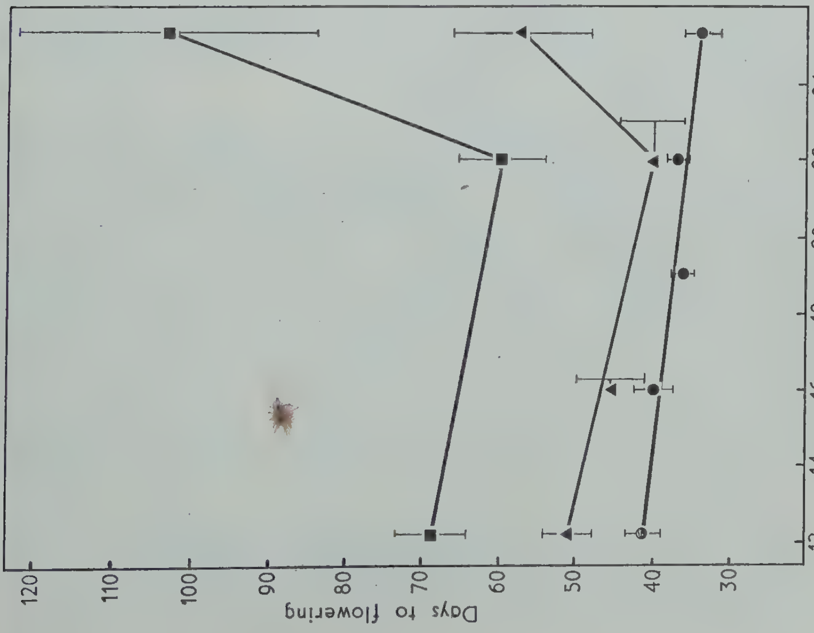

FIG. 6

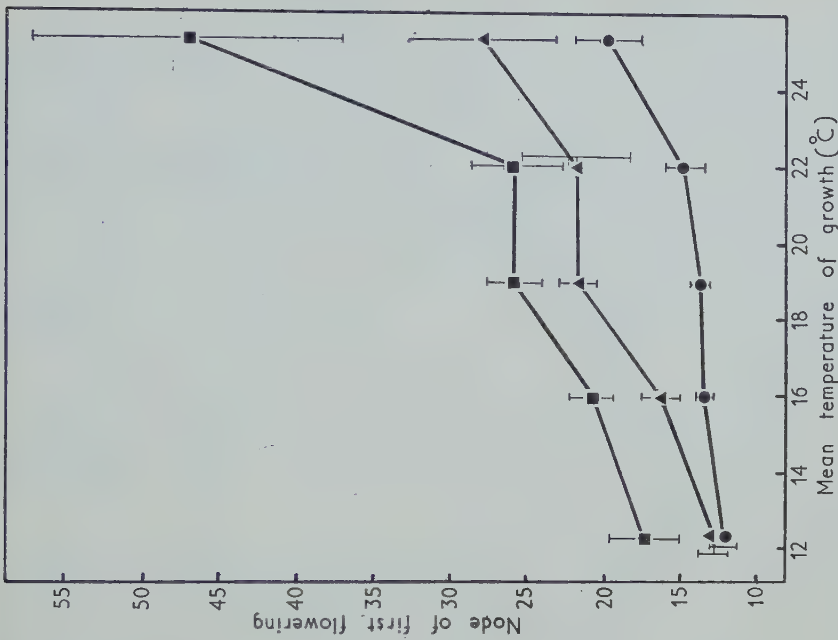

FIG. 7

FIGS. 6 and 7. The effect of temperature on the time (Fig. 6) and node (Fig. 7) of first flowering in French winter beans grown in photoperiods of 8 hours (■), 16 hours (▲), or continuous light (●).

natural light intensity. For this reason, only the node of first flowering results are given for the plants sown during the winter months.

In continuous light there is a slight but significant decrease in the time to floret appearance as the temperature of growth rises from 17°–10° C. to 30°–23° C. When a dark period is present in the photocycle, however, the decrease in time to floret appearance with rising temperature of growth is reversed above 26°–20° C., with the result that highly significant delays in flowering occur at the highest temperature, especially in 8-hour photoperiods, in which conditions flower initiation is also far more variable than it is in longer photoperiods and at lower temperatures. A similar result was obtained by Steinberg and Garner (1936) and by Owen *et al.* (1940) with *Beta vulgaris*, another vernalizable long-day plant. Such a result suggests that a reaction inhibitory to early flowering may occur at high temperatures, with greater effect the shorter the photoperiod and hence possibly only in the dark.

The node of first flowering results in Fig. 7 do not show such a clear pattern, since in all three photoperiods there is an increase in the node of flower initiation as the temperature rises. However, the increase is most pronounced at the highest temperature in short days.

Effect on floret appearance. The effect of temperature on the time and node of flower initiation in four stocks of *V. faba*, when grown in 8-hour photoperiods, is shown in Figs. 8 and 9. There were 8–10 plants in each treatment. The French winter, Suffolk Red spring, and Dutch Weir beans were grown in natural daylight at temperatures 6°–7° C. higher than the night temperatures, except in a few of the Suffolk Red spring bean treatments in which relatively higher night temperatures were given. With the exception of one group that was grown in natural daylight at 23° C. and a night temperature of 17° C., the Bullocks winter beans were grown under artificial light with no alternation of day and night temperatures.

Even in short days, the very early Dutch Weir bean shows no delaying effect of high growth temperatures on progress to flowering, whereas the other stocks, including the early flowering Suffolk Red spring bean, do so. The optimal mean temperature for early flower initiation in Bullocks winter beans (17° C.) may be rather lower than those for French winter and Suffolk Red spring beans because the same mean temperature of growth represents a rather higher night temperature in the former case. In all three stocks there is evidence of a reaction which is strongly inhibitory to early flower initiation above about 18°–22° C., and which increases in its inhibitory action as the temperature of growth rises above this range. That this reaction is of much less consequence when the dark period is short or absent, as previously shown to be the case in French winter beans, was also found in Suffolk Red spring beans.

The results for node of first flowering given in Fig. 9 also suggest the operation of a reaction which delays flower initiation at high temperatures in all except the Dutch Weir beans. But whereas there is an increase in the time to floret appearance in all stocks as the temperature falls below 17° C., there

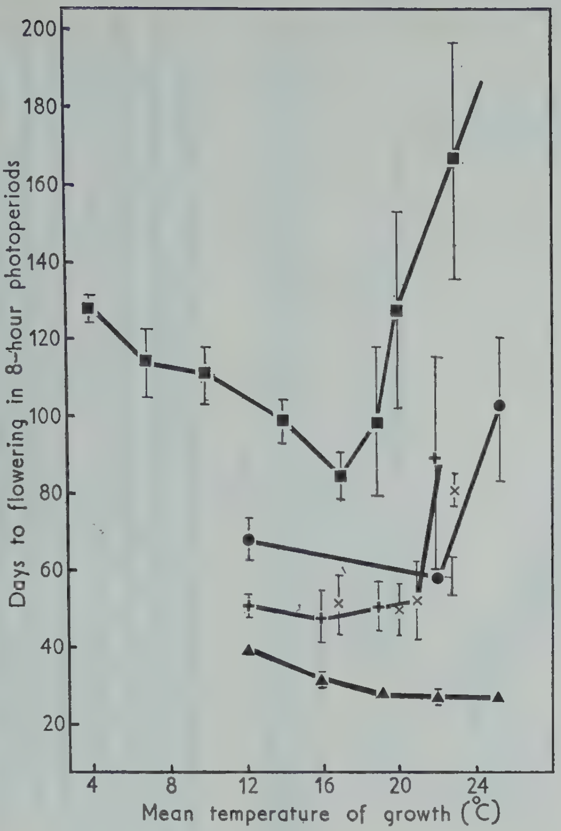

FIG. 8

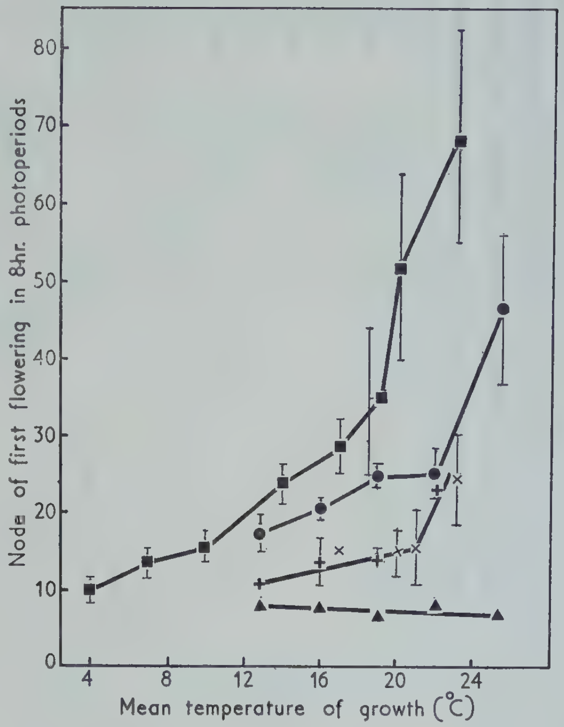

FIG. 9

FIGS. 8 and 9. The effect of temperature on the time (Fig. 8) and node (Fig. 9) of first flowering in four stocks of *Vicia faba*: Dutch Weir spring beans (▲); Suffolk Red spring beans at night temperatures 6° C. below day temperatures (+), and in constant temperatures (×); French winter beans (●); Bullocks winter beans (■).

is on the other hand a reduction in the node of first flowering, except in the Dutch Weir beans.

The discrepancy between the results in terms of days to floret appearance and those in terms of node of first flower initiation in Bullocks winter beans may be seen in Fig. 10. The close agreement between the node of first flowering results and the rates of node formation at temperatures below 19° C. suggests that to some extent the low nodes of first flowering at low temperatures may be merely a reflection of slower rates of node formation rather than of specific effects on flower initiation.

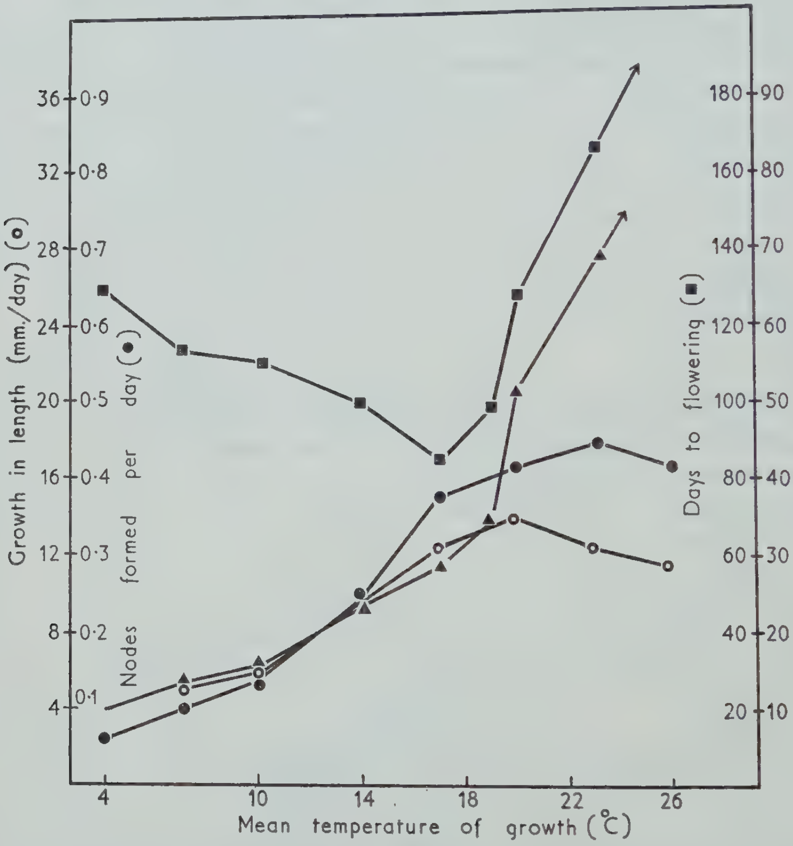

FIG. 10. The effect of temperature on Bullocks winter beans grown in 8-hour photoperiods. The vertical scales from left to right are for: growth in height in mm. per day (o); number of nodes formed per day (●); days to floret appearance (■); number of nodes to first flowering (▲).

Night temperature and flowering. The results already quoted for French winter beans suggested that the inhibitory action of high temperatures on flowering might be confined to the diurnal dark periods. Two moderately early-flowering stocks of *V. faba* were therefore grown at a series of night temperatures in combination with photoperiods at one temperature. There were 5 plants in each treatment. The Ferry Morse beans were grown under artificial light of 500–700 foot-candles intensity for 16 hours at phototemperatures of either 17° or 26° C. and were then transferred to the dark rooms. The Suffolk Red spring beans were grown in natural light for 8 hours at phototemperatures of 17°, 20°, or 23° C. and were then transferred to the required night temperatures although they continued to be illuminated artificially for another 8 hours. The results of both experiments are given in Fig. 11.

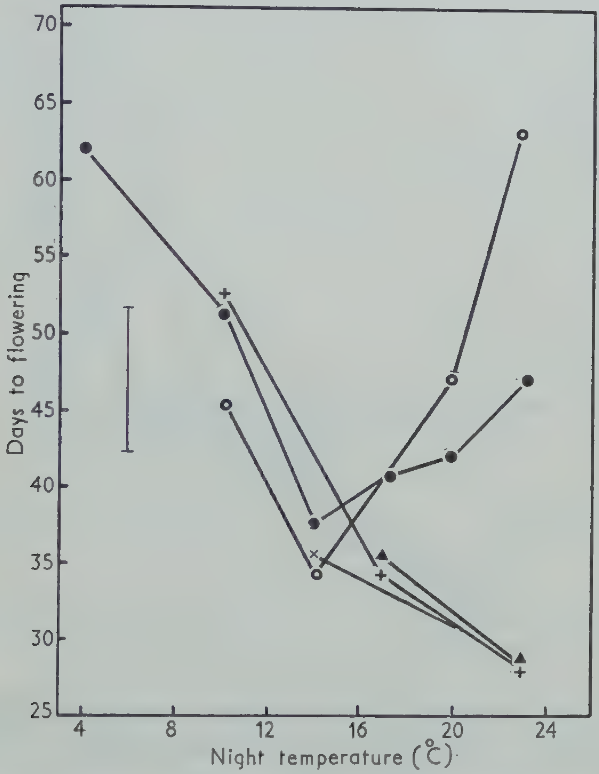

FIG. 11. The effect of night temperature on the time to floret appearance in two stocks of beans grown in 16-hour photoperiods: Suffolk Red spring beans, grown at day temperatures of 23° C. (▲), 20° C. (+), 17° C. (×); Ferry Morse beans, grown at day temperatures of 26° C. (o) and 17° C. (●).

The Suffolk Red spring bean seed had been harvested from a plant grown in a relatively cool temperature and this, in conjunction with the long day length, may account for the absence of any high temperature inhibition. The Ferry Morse stock, on the other hand, shows a pronounced night temperature optimum at 14° C. for both phototemperatures, with progressively delayed floret appearance at higher night temperatures. There is also, however, a significant accentuation of this delay at the higher phototemperature, which suggests that the inhibitory effect is not entirely restricted to the dark period.

Inductive temperature treatments. Low temperatures throughout the life cycle have been shown to delay floret appearance even in winter beans. On the other hand, published reports indicate that seed vernalization may hasten flowering in winter beans, and an experiment was designed to examine the effects of short periods at various temperatures on flower initiation in Bullocks winter beans. There were 8 plants in each treatment and the control group was grown in natural daylight for 8-hour photoperiods at 23° C. with transfer to dark rooms at 17° C. for 16 hours. The treated plants, after sterilization of the seeds in a hypochlorite solution followed by imbibition at room temperatures for 4 hours, were grown at each temperature available from 4° to 26° C., for 1, 2, 4, 8, or 16 weeks, in 8-hour photoperiods, before transfer to the control conditions.

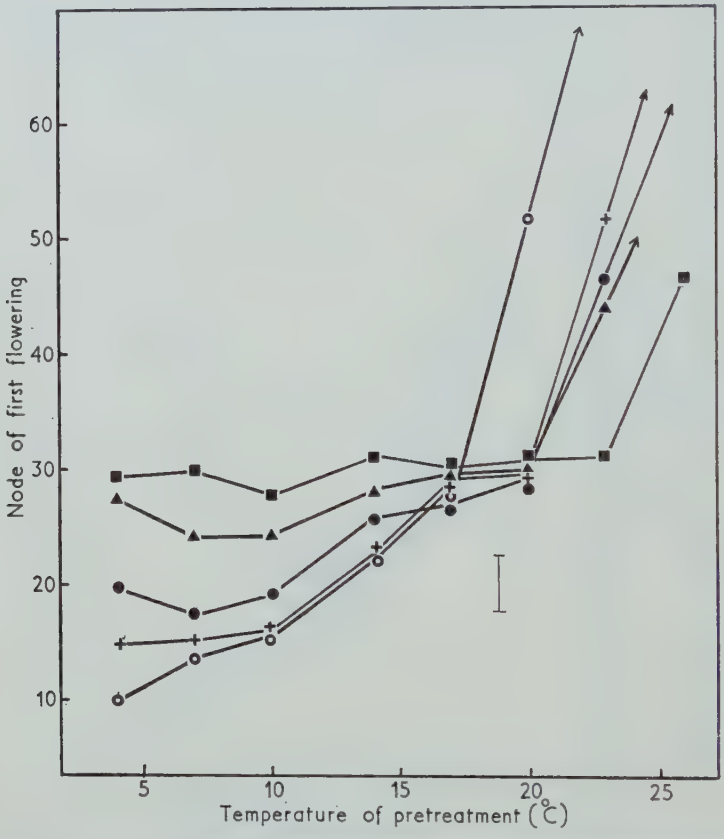

FIG. 12. The effect of pretreatment temperature and time on the node of first flowering in Bullocks winter beans grown in 8-hour photoperiods at 23°–17° C.: after 7 days (■), 14 days (▲), 28 days (●), 56 days (+), or 112 days (o) of pretreatment.

The results of the experiment, in terms of node of first flowering, are given in Fig. 12. By far the most striking result of one week of pretreatment at the various temperatures is the highly significant increase in the node of flower initiation at 26° C. Two weeks of pretreatment at 26° C. inhibited flowering in some of the plants in this stock of winter beans, while 2 weeks at 23° C. considerably delayed it. After 16 weeks of pretreatment there is even at 20° C. a significant increase in the node of first flowering over that of the control group. On the other hand, pretreatment at temperatures lower than 17° C. has, at each temperature, progressively lowered the node of flower initiation with time of treatment, most markedly at 10° C. after 1 week, at 7° C. after 4 weeks, and at 4° C. after 8 weeks of pretreatment. Such a result is essentially similar to those obtained by Stout (1946) with *Beta vulgaris*, by Purvis (1948) with winter rye, and by Lang (1951) with *Hyoscyamus niger*, and implies a positive temperature coefficient for the whole vernalization process within the effective temperature range.

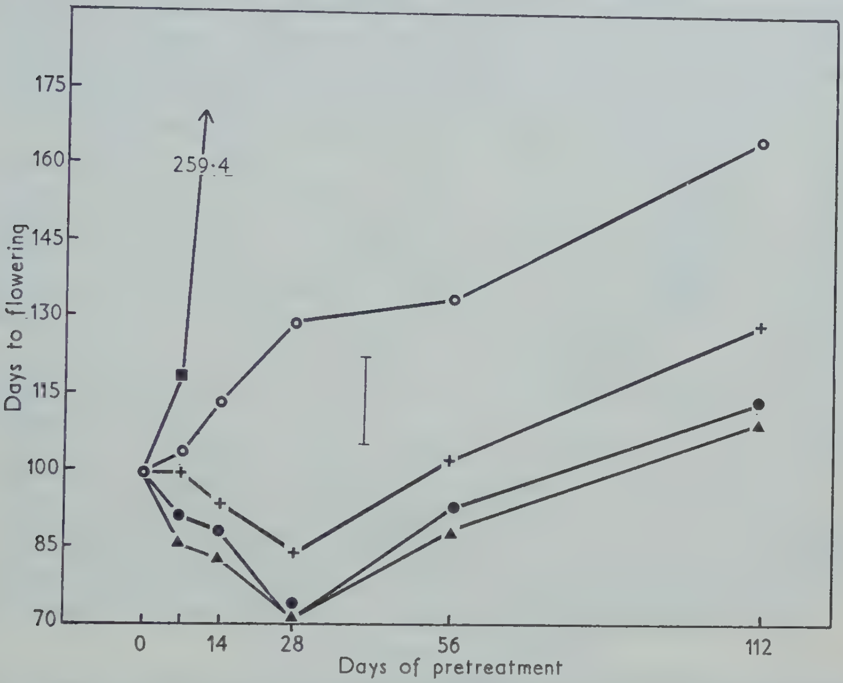

FIG. 13. The effect of pretreatment temperature and time on the time to floret appearance in Bullocks winter beans grown in 8-hour photoperiods at 23°–17° C.: pretreatment at 26° C. (■), 23° C. (o), 10° C. (▲), 7° C. (●), and 4° C. (+).

In Fig. 13 the effects of pretreatment with the highest and lowest temperatures used are shown in terms of time to floret appearance. Whereas pretreatment at 4° C. ultimately resulted in the greatest lowering of the node of first flowering, that at 10° C. has consistently resulted in the greatest reduction in time to flower primordia appearance. Tanaka (1936) likewise found that seed vernalization at 10° C. hastened flowering in *V. faba* more than at 5° C. At 4° C. there appears to be a lag of at least 1 week before any acceleration of flower initiation is evident, and this to some extent accounts for the reduced vernalization effect at 4° C. compared with that at 7° and 10° C.

The effect of 1 and 2 weeks pretreatment at 26° C. is far more striking than that of the low temperatures. Flowering after 2 weeks at 26° C. was incomplete, only 5 of the 8 plants producing flower primordia, and then only after an average of 259·4 days. Pretreatment at 23° C. also delayed flowering considerably, but the inhibitory action is obviously much less effective than at 26° C. Such results are very different from those obtained by Robitzsch (1938), who found no effect of any of his preliminary treatments, even of one week at 32° C. in the dark, on flowering in any of the bean stocks used by him. However, his results refer to full flower development, not to the date of flower primordia appearance. Moreover, the data given for his latest flowering stock (Wadsacks) indicate that it was much earlier flowering than either the French or Bullocks winter beans used in the present study, since it reached full bloom in less than 70 days in 8-hour photoperiods and hence was unlikely to show any marked response to inductive temperature treatments.

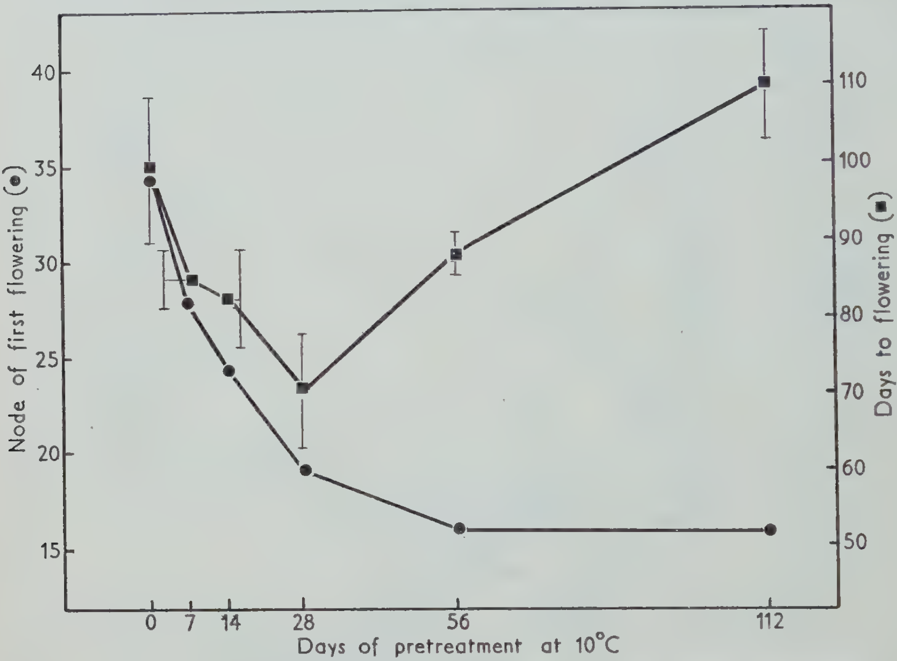

FIG. 14. The effect of time of pretreatment at 10° C. in 8-hour photoperiods on the node (●) and time (■) of first flowering in Bullocks winter beans subsequently grown at 23°–17° C. in 8-hour photoperiods.

In Fig. 14 the results for the pretreatments with 10° C. are given in terms of nodes and times to floret appearance. Both show significant reductions for pretreatment periods up to 28 days, but whereas longer periods at 10° C. result in a further reduction in the node of first flowering, they cause a considerable delay in the appearance of flower primordia. The node of first flowering data suggest that the vernalization response was approaching saturation after 56 days, by which time the plants at 10° C. had differentiated about

12 nodes, yet flower initiation did not take place until a mean of 15·8 nodes was formed. This suggests that a process subsequent to the vernalization process must take place before flower induction can occur, and if the temperature coefficient of this post-vernalization process were less than that for node formation, the node of first flowering would continue to fall even after saturation of the vernalization response. This would explain the discrepancy between the results for time and those for node of flower initiation after pretreatments at low temperatures. It implies, moreover, that the low nodes of first flowering after extended periods at low temperatures are to some extent due to effects on the rate of node formation rather than to specific effects on flower initiation.

Removal of the high-temperature inhibition. The results given in the previous section indicated a slight lag in the vernalization process at 4° C. This could be due to limitations imposed by the slowness of some metabolic process antecedent to vernalization, in which case an initial period at normal temperatures might increase the effectiveness of a period at 4° C. in hastening flower initiation. On the other hand, an initial period at high temperatures could delay flowering unless cold treatment overcame the effects of high-temperature inhibition.

Groups of 20 seeds of Bullocks winter beans were germinated for 7 days in 8-hour photoperiods at temperatures from 17° to 30° C.; half of each group of seedlings was then grown in natural daylight for 8-hour photoperiods at 23° C. followed by 16-hour dark periods at 17° C., while the other half of each group was given 4 weeks at 4° C. in 8-hour photoperiods before transfer to 23°–17° C. The results of the experiment are given in Table 1.

TABLE 1

Time and Node of Floret Appearance in Bullocks Winter Beans, after Pretreatment by High Temperatures for 7 days at Germination, followed in some Cases by 4 Weeks at 4° C. before Transfer to the Control Conditions

	Control	7 days pretreatment at				
	23°–17°	17°	20°	23°	26°	30° C.
(*a*) Unvernalized						
Days to floret appearance	99·2	94·7	96·4	103·1	117·5	No flowering
Node of first flowering	30·6	30·0	30·8	31·0	46·7	
(*b*) Vernalized						
Days to floret appearance	84·1	78·5	79·2	82·2	80·7	86·2
Node of first flowering	19·5	17·8	18·2	19·8	19·5	21·3

Significant difference at $P = 0{\cdot}05$, 5·5 days.

It is clear that the delaying effect of high germination temperatures on flower initiation can be removed by a subsequent cold treatment of 4 weeks at

4° C. On the other hand, pretreatment at 17° C. for 7 days significantly increased the effectiveness of 4 weeks at 4° C. in hastening flower initiation.

Effect of plant age on the response to cold treatment. The preceding results suggest that the effectiveness of a given period of cold treatment in hastening flower initiation may vary with the age of the plants treated. Bullocks winter beans were sown under 8-hour photoperiods of natural light at 23° C., with dark periods at 17° C., and were removed in groups of 8, after 7, 14, 28, and 56 days, to 8-hour days under artificial light at 4° C., for 7, 14, or 28 days, and then returned to the control conditions. The results of the experiment are given in Fig. 15, together with the results for the same treatments of newly imbibed seed (from Fig. 13).

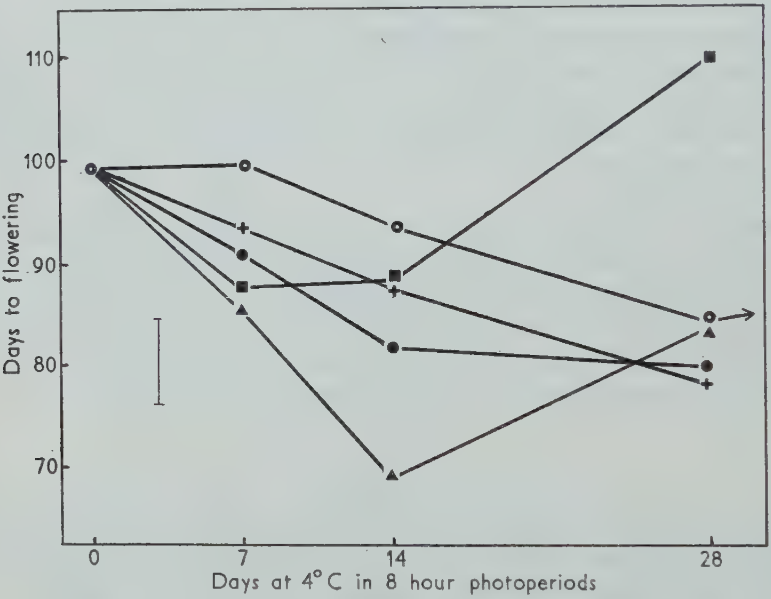

FIG. 15. The effect of plant age on the response to vernalization at 4° C. Cold treatment begun at germination (o), or after 7 days (+), 14 days (●), 28 days (▲), or 56 days (■) at 23°–17° C.

In the seedlings of all ages there appears to be no lag in the vernalization reaction at 4° C. as there is at germination. Moreover, the accelerating effect of short periods of cold treatment on flower initiation increases considerably with increase in the age of the plant up to 4 weeks. There is also a tendency for the optimal length of cold treatment to fall with increasing plant age, from 28 days for the youngest seedlings to 14 days for 4-week-old plants and 7 days for plants 8 weeks of age.

The effect of temperature during pod development. The plants used in the pilot experiments were left to set seed under the conditions in which they had initiated flowers. When the pods were fully ripened the seeds were harvested, and bulked lots of 20 seeds of Suffolk Red spring beans from each temperature of pod development were then germinated at 17° C. for 2 weeks and divided into two groups, one being grown at 20°–14° C. and the other at 26°–20° C., under natural daylight for 8-hour photoperiods. The results are given in Table 2.

TABLE 2

The Number of Days and Nodes to First Flower Initiation in Suffolk Red Spring Bean Plants Grown from Seed Developed at various Temperatures

Temperature of growth		Temperature of pod development				
		10°	17°–10°	20–°14°	23°–17	26°–20° C.
20°–14° C.	days	32·3	39·0	40·5	38·0	62·6
	nodes	7·7	10·6	12·3	10·9	19·4
26°–20° C.	days	26·7	33·1	37·0	31·0	66·4
	nodes	7·7	10·4	11·7	10·9	24·6

Significant difference at $P = 0{\cdot}05$, 7·6 days.

Once again the most striking effect is that of the highest temperature. Flower initiation in plants grown from seeds which developed at 26°–20° C. was delayed very significantly compared with that in plants from seeds which developed at intermediate temperatures. At the other extreme, plants from 10° C.-seed were only slightly advanced in flower primordia appearance, as would be expected in an early flowering stock. Gregory and Purvis (1938) and others have already shown that cold treatment is effective during seed development, and the results given above indicate that the high-temperature process which is inhibitory to flower initiation in *V. faba* may also be active during seed development. The results also suggest that some of the variability in flowering behaviour of plants grown from commercial seed could be due to variations in field temperatures during the long period of pod development.

SOME INTERACTIONS OF TEMPERATURES DURING SEED DEVELOPMENT, GERMINATION, AND GROWTH

It has been shown that both the low-temperature acceleration and the high-temperature inhibition of flowering may occur during any stage in the life cycle of *V. faba*, that the effectiveness of a given period of cold varies with the age of the plant, and that cold-temperature treatment soon after germination can apparently remove the inhibitory effects of high temperatures given at germination. A factorial experiment was designed to examine further some of the interactions of three levels of temperature during seed development, during germination, and during growth of the plants. Suffolk Red spring beans were used, there were 5 plants per treatment, and all germination and growth took place in 8-hour days. The seed-development temperatures chosen were 10°, 20°–14°, and 26°–20° C., the seed being taken from the bulked harvests of plants which had initiated flowers and matured pods under these temperatures. The germination treatments were 4 weeks at 4° C., 2 weeks at 17° C., and 1 week at 23° C., under artificial illumination. The growth temperatures were 17°–10°, 20°–14°, and 26°–20° C., under natural light. Certain features of the experiment need comment before the results are considered. The bean stock used was an early-flowering one, and only limited responses to the various temperature treatments could be expected; it was, however, the only stock for which a sufficient harvest of seeds set in the Earhart Laboratory was available. The number of plants per treatment was limited by the small harvest of seed at 26°–20° C., at which temperature few pods ever reached maturity, and at 10° C., where pod development was extremely slow though abundant. And had the results of some of the experiments already discussed been available at the time, germination temperatures other than those used would have been selected to give more striking differences.

The results in terms of node of first flowering (Fig. 17) show a more or less progressive increase in node of initiation with increase in temperature at any stage of the life cycle. Plants from seed harvested at 10° C. show a very low average node of first flowering whatever the subsequent germination and growth temperatures: about half of those germinated at 4° C. flowered at the sixth node, the lowest figure obtained in any of the present experiments.

The results in terms of days from germination to floret appearance (Fig. 16) are rather different. As was to be expected in an early flowering stock, the acceleration of flowering in the 10° C.-harvested seed under all conditions of germination and growth is much less marked than the delay in floret appearance in plants from seed harvested in 26°–20° C. Flower initiation in plants from the 26°–20° C. seed is delayed most when germination and growth also take place at high temperatures, least when both are at low temperatures. Even so, it is clear that germination at 4° C. and growth at 17°–10° C. has not completely removed the delay in flowering due to seed development at 26°–20° C. However, the 4° C.-germinated plants from 26°–20° C. seed do behave like plants from 10° and 20°–14° C. seed in that an increase in the temperature of growth slightly hastens their initiation of flowers whereas it delays that of the plants from unvernalized 26°–20° C. seed. Only under high temperatures of germination and growth do differences between plants from the 10° C. and 20°–14° C. harvests become evident. But in both, germination at 23° C. has no delaying effect on flower primordia appearance when it is followed by a cool temperature of growth, and only a slight delaying effect at higher growth temperatures. Had the germinations been carried out at 26° C. instead of at 23° C., more pronounced differences between the 10° C. and the 20°–14° C. seed might have emerged.

It is clear that the accelerating influence of periods at temperatures lower than 14° C., and the delaying effect of temperatures above 23° C. or so, on the rate of appearance of flower primordia may operate at almost any time in the life cycle of plants of *V. faba*, and that subsequent treatment with the one does to some extent remove the effects of the other. A high growth temperature, however, although it may add greatly to the delay in flowering when it

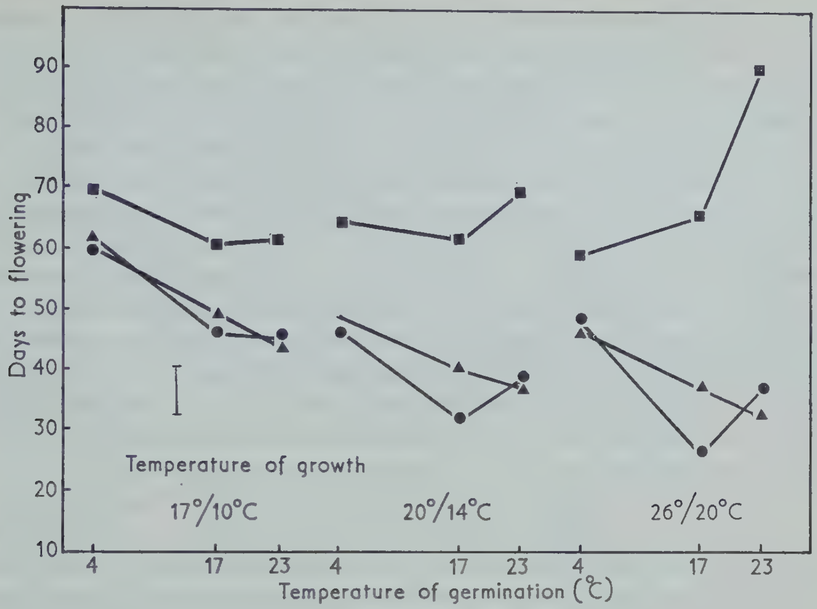

FIG. 16.

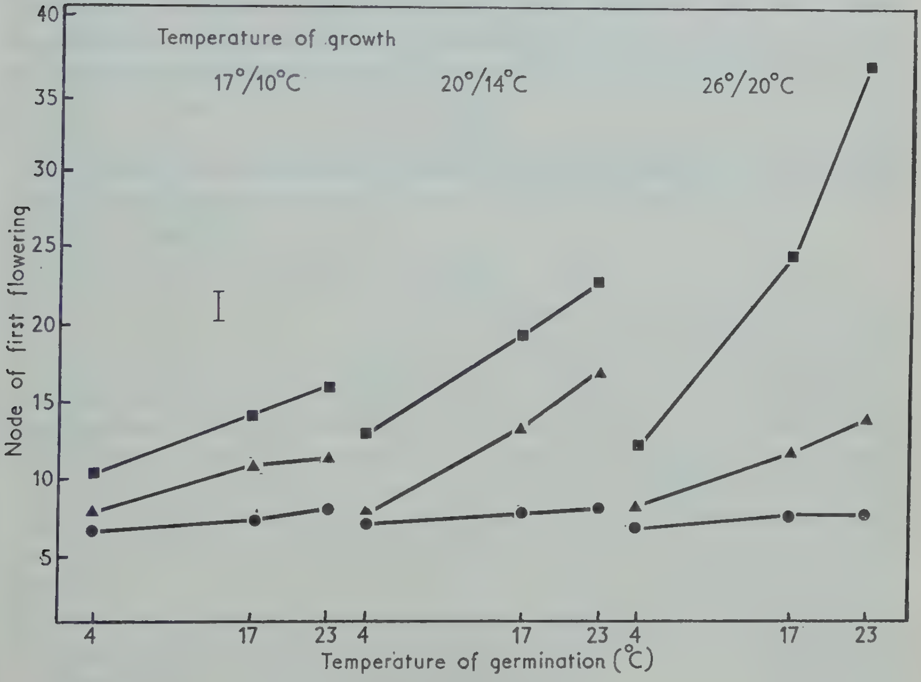

FIG. 17.

FIGS. 16 and 17. The effects of temperature during seed development, germination, and growth, on the time (Fig. 16) and node (Fig. 17) of first flowering in Suffolk Red spring beans grown in 8-hour photoperiods: plants from seeds developed at 10° C. (●), 20°–14° C. (▲), and 26°–20° C. (■).

follows high temperatures of seed development and germination, actually hastens flowering when it has been preceded by a low or moderate temperature of germination, especially when seed development has also occurred at a low temperature. This suggests that in this stock of *V. faba*, provided high-temperature inhibition is absent during seed development and germination, a state is reached in which high temperatures are no longer inhibitory to flower initiation, and in which the processes subsequently leading to initiation are actually favoured by them.

One further point may be noted; comparison of the results in Figs. 16 and 17 suggests that node formation in plants from 10° C. seed was much slower, and that in plants from 26°–20° C. seed rather faster, than in those grown from 20°–14° C. seed, an inductive effect that would confuse to some extent the interpretation of the node of first flowering results. It was also found that the growth-rates of plants from the 26°–20° C. seeds were considerably higher than those of plants from seeds set at lower temperatures.

SOME VERNALIZATION RESPONSES

Although the early-flowering spring beans do not usually show earlier flower initiation in response to periods at a low temperature, they may do so under conditions adverse to early initiation. Thus Suffolk Red spring bean plants from seed harvested at 26°–20° C. and grown at that temperature responded to low-temperature treatment (Fig. 16), and again in one of the pilot experiments (Table 3) with commercial seed the same stock gave a sig-

TABLE 3

The Effect of Periods at Low Germination Temperatures on the Number of Days from Imbibition to the Appearance of Florets in several Bean Stocks

		Vernalization treatment				
	Photo-period	None	4° C.			10° C.
Growth temperature			10	20	30	30 days
(*a*) Suffolk Red spring beans						
20°–14° C.	8	51·8	68·5	—	71·0	63·5
26°–20° C.	8	78·3	64·3*	67·5	67·8	63·8*
26°–20° C.	16	47·3	61·0	64·3	67·0	65·0
(*b*) French winter beans						
23°–17° C.	8	79·0	—	—	81·0	—
23°–17° C.	16	58·9	—	—	60·5	—
(*c*) Gartons Giant winter beans						
17°–10° C.	8	99·0	—	—	—	—
17°–10° C.	16	79·0	—	—	—	—
20°–14° C.	8 and 16	X	—	—	—	—
23°–17° C.	8 and 16	X	—	—	—	—
26°–20° C.	8	X	X	X	75·8	X
26°–20° C.	16	X	(90)	75·0	75·3	79·8

X = no flowering.
* Significant difference from controls at $P = 0{\cdot}05$.

nificant vernalization response when grown in short days at high temperatures. In long days at 26°–20° C., and in short days at cooler temperatures, even brief periods at low germination temperatures caused a delay in flower initiation.

On the other hand, some of the winter beans did not always show a response to low-temperature treatment at germination, as may be seen in Table 3 for the French winter beans. In this stock, although the time to floret appearance was not reduced by low-temperature treatment, the node of first flower initiation was significantly ($P = 0{\cdot}01$) lowered in all cases. However, when node formation was followed in the treated and untreated plants it was found that in all conditions the rate of node formation subsequent to cold treatment was 10–20 per cent. less than that in the control plants. This inductive effect of vernalization on the rate of node formation was statistically significant in four of the five groups of plants in which it was measured, and accounted for 27–51 per cent. of the lowering in node of first flower initiation.

The latest-flowering bean stock used, Gartons Giant winter bean, showed no flower initiation in either long or short days except at the lowest temperature of growth, 17°–10° C. (Table 3), at which temperature initiation occurred in both photoperiods, though more rapidly in the longer days. In long days at the highest growth temperature used, 26°–20° C., 1 plant initiated flower primordia after receiving only 10 days' treatment at 4° C., and all initiated them after 20 days at 4° C., whereas in short days at 26°–20° C. 30 days at 4° C. were necessary for the induction of flowering.

An experiment was carried out in which Bullocks winter bean plants had their roots maintained at temperatures of 10°, 20°, or 30° C. while their shoots were exposed to ambient temperatures of 17°–11° C. or 30°–23° C. under natural short day-lengths, but there was no evidence of any hastening effect of low root temperatures on flower initiation, nor of any retarding effect of high root temperatures.

EFFECT OF SOME CHEMICAL TREATMENTS ON THE TIME TO FLOWER INITIATION

Seeds of the late-flowering French winter beans were treated with a variety of substances in an attempt to obtain an acceleration of flowering. Several treatments, such as those with 0·3 M. mannitol, gave a significant lowering of the node of first flowering without affecting the time to floret appearance, and such effects must be presumed to be mainly on the rate of node formation. Only the very few treatments in which statistically significant reductions were obtained in the time from imbibition to floret appearance will be mentioned below.

The technique used was to soak all seeds in water at room temperature for 4 hours, after a cut had been made in the testa opposite the hilum to facilitate imbibition. The seeds were then placed in small bottles containing either water or the treatment solutions, and these were mechanically shaken in the dark at 17° C. for 12 hours, after which the seeds were sown. There were 8–9 plants in each treatment.

The results of an early experiment are given in Table 4*a*. The diffusate, obtained from seeds of the very early flowering Dutch Weir spring beans in the manner described by Highkin (1955), hastened flower initiation slightly under all conditions, but only to a significant extent in the cooler temperature under long days. The nucleosides, a 10^{-3} M. solution of adenosine, guanosine, uridine, and cytidine, effected a highly significant acceleration in the appearance of florets in both long and short days at the cooler temperature.

In a further experiment, the results of which are given in Table 4*b*, seeds which had imbibed water or the nucleoside solution were kept at 4° C. for 7 days before planting out. As would be expected from previous results, this short period at 4° C. has not hastened floret appearance in the controls, but neither has it noticeably increased the accelerating effect of the nucleosides, as it might have done had they been involved in the low-temperature process. Solutions of the four individual nucleosides mentioned, of various nucleotides, of kinetin, and of several amino-acids were tried at various concentrations, but none gave a significant lowering of the time to floret appearance, although guanosine did give a consistent one.

TABLE 4

The Effect of Various Chemical Treatments on the Number of Days from Imbibition to Floret Appearance in French Winter Beans

Growth temperature	20°–14° C.		23°–17° C.	
Photoperiod	8	16	8	16
(*a*) Control	75·3	55·1	79·0	58·9
Diffusate	70·2	51·0*	74·7	53·2
Nucleosides	64·5†	47·9‡	83·8	55·2
(*b*) Control	—	54·8	—	—
Nucleosides	—	45·3*	—	—
(*c*) Control	—	62·3	—	—
10^{-3} M. 2′, 6′, diaminopurine	—	61·4	—	—
10^{-3} M. 5, hydroxyuridine	—	61·8	—	—
10^{-4} M. canavanine	—	64·2	—	—
10^{-4} M. thiouracil	—	71·8†	—	—
10^{-5} M. thiouracil	—	68·5‡	—	—

Significant difference from control: *, $P = 0{\cdot}05$; †, $P = 0{\cdot}02$; ‡, $P = 0{\cdot}01$.

In Table 4*c* are given some of the results of an experiment in which seeds were imbibed with solutions of purine and pyrimidine analogues before vernalization at 4° C. for 4 weeks. Three of the analogues had no effect on the time to floret appearance, whereas thiouracil significantly delayed it at all concentrations used. However, the thiouracil treatments also affected growth adversely, and in view of the lack of effect by the other analogues, and by the nucleoside solution on the cold-treatment response, it seems unlikely that nucleic acid components are specifically involved in the vernalization reaction in *V. faba*.

POST-INDUCTIVE FLOWER DEVELOPMENT

Noye (1945) suggested that although flower initiation in *V. faba* may take place in short days, full floral development only occurs in long days. Her conclusion was confirmed for all the broad bean stocks used in the present experiments.

Under continuous light there is scarcely any delay in flower expansion after initiation has occurred: in French winter beans full flower development occurred at the first node of flower initiation, and in Bullocks winter beans at an average of 2·5 nodes later in 23°–17° C. At the same temperature in 16-hour photoperiods there was a lag of 9·7 nodes from initiation to floral expansion in Bullocks beans, with no expansion occurring in 8-hour days. In 16-hour photoperiods the delay in floral expansion depends to some extent on the temperature, being greater at higher temperatures, as may be seen in Table 5.

TABLE 5

The Number of Nodes between Flower Initiation and Flower Expansion in Suffolk Red Spring Beans in 16-hour Photoperiods. 10–12 Plants per Treatment

Growth temperature	17°–10°	20°–14°	17°–17°	23°–17°	20°–20°	26°–20°	23°–23°	30°–26° C.
Mean node lag	0	1·9	2·8	6·3	3·7	6·1	7·6	8·2

Plants in all greenhouses of the Earhart Laboratory began to develop full flowers during the first week in April when the natural day-length at Los Angeles was about 12·5 hours, or 13·5 hours with official twilight added. However, none of the French winter beans grown in 12-hour photoperiods at 23°–17° C. bore any fully developed flowers, which suggests that the normal critical photoperiod for floral expansion in *V. faba* is about 12–13 hours.

Fully developed flowers did occur on two Suffolk Red spring bean plants in 8-hour days, however, and the exceptions are of interest in that they were both plants from seeds which had developed at 10° C. and germinated at 4° C. This suggests that low-temperature treatment may to some extent compensate for the lack of a sufficiently long photoperiod for floral expansion.

DISCUSSION

Flower initiation in *V. faba* can take place over a wide range of temperatures and photoperiods, and progress towards it is concurrent with growth under most conditions. Since the environmental conditions which affect it also affect the growth-rates of the plants, it is often difficult to evaluate the specific effect of particular environmental conditions on flower initiation in *V. faba*, and both time and node of flowering data must be considered.

In all stocks examined, increase in photoperiod length increased the rate of leaf appearance as well as the rate of flower initiation, with the result that in the earliest-flowering stock the node of first flowering was unaffected by photoperiod. In the later-flowering stocks, however, flowering was accelerated relative to node formation by increase in photoperiod length, and these stocks of *V. faba* may be considered quantitative long-day plants.

The response to temperature is more complex. At least three processes affected by temperature appear to be involved in the initiation of flowering: one, the vernalization process, is favoured by low temperatures, the others, dark inhibition and a post-vernalization process, by high temperatures.

Even early-flowering stocks of *V. faba*, such as Suffolk Red spring beans, may respond to vernalization when the plants are subsequently grown under short days and high temperatures, while late-flowering stocks, such as Gartons Giant winter beans, require vernalization if they are to flower at all under such conditions. Cold treatment is effective at germination, and vernalization can occur in the developing embryo to such an extent that subsequent high temperatures at germination and growth cannot delay flowering. However, periods at normal growing temperatures before cold treatment removed the lag in the vernalization response at 4° C., hastened the approach to saturation of the response, and considerably increased the acceleration of flower initiation in winter beans by cold treatment. These results imply that the rate of the vernalization process in germinating seeds of *V. faba* at 4° C. is limited by an antecendent process which is favoured by higher temperatures. No lag in the vernalization response was evident at 10° C., which suggests that at that temperature the precursor process does not limit the rate of vernalization. It may be for this reason that vernalization at 10° C. was apparently more rapid than vernalization at 4° C., i.e. that it is not so much the vernalization process itself as the antecendent process which increases in rate with increase in temperature.

Once the vernalization response is saturated, further exposure of *V. faba* plants to low temperatures only delays flower initiation, although it reduces the node of first flowering. This discrepancy could be explained if there was a process following vernalization which hastened flowering, and which increased in rate with increasing temperature, but to a lesser extent than the rate of node formation. The existence of inductive processes following vernalization is suggested by the analysis of the results given in Fig. 14. After saturation of the vernalization response at 10° C., the plants had differentiated about 12 nodes on the average, yet flower initiation did not take place until a mean of 15·8 nodes was formed. The extent of the delay in flowering after extended periods at 10° C. indicates that the post-vernalization processes were about one-half to two-thirds as rapid at 10° C. as at 23°–17° C., whereas node formation was only about one-third as rapid (Fig. 10).

The inhibitory effect on flower initiation of long dark periods at high temperatures is known for many long-day plants. The results given in Fig. 6 suggest that in *V. faba* the inhibitory effects of high temperatures are largely confined to the diurnal dark periods, although the results given in Fig. 11 suggest that they may not be entirely so. With the Ferry Morse stock temperatures above 14° C. during 8-hour dark periods delayed flowering, while with the earlier flowering Suffolk Red spring beans 8-hour dark periods at

23° C. were not inhibitory although 16-hour dark periods at 23° C. were. The inhibitory effect of dark periods on the flowering of *V. faba* also depends on the period of exposure to high temperatures. Thus, flowering in Bullocks winter beans was completely inhibited after 2 weeks at 26° C. or 16 weeks at 23° C., and was delayed by 1 week at 26° C. or 2 weeks at 23° C.

The inhibitory effect of high temperatures on early flower initiation can be effected while the seeds are developing in the pod, as well as during germination or subsequent growth of the plants, and, if no periods at low temperatures intervene, the effect of high temperatures at various stages in the life cycle are more or less additive, there being no apparent upper limit to the effect short of prevention of flower initiation altogether. The additive nature of the high-temperature inhibition suggests that the inhibitor may accumulate over long periods at the site of the flower promoting processes.

Late-flowering stocks of *V. faba* could have either a more efficient dark inhibitory process or a lower efficiency in their vernalization or other flower-promoting processes. That it is not the former is suggested by the fact that the slope of the response of late-flowering stocks to photoperiod length was scarcely different from that of the early-flowering stocks. The relation between the vernalization and the inhibitory processes is unknown. That early-flowering stocks of *V. faba* only respond to vernalization in short days at high temperatures, and that in late-flowering stocks the effects of the dark inhibitory reactions can be overcome by subsequent vernalization while adequate vernalization renders the plant no longer susceptible to dark period inhibition, could be explained by the dark inhibitor competing with the product of the vernalization process as a substrate for an enzyme involved in a later process accelerating flower initiation.

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Some effects of *Plasmodiophora brassicae* Woron. on the growth of the young cabbage plant

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With seven Figures in the Text

ABSTRACT

When clubroot galls developed on cabbage inoculated with *Plasmodiophora brassicae* Woron., the distribution of dry matter in the plant was altered. As soon as clubbed roots were visible the stem: root ratio diminished with time until the clubs rotted. After clubs appeared the increase in root weight was nearly all from growth of the clubroot gall and the rate of growth of tops correspondingly diminished. Succulence of the shoot, water content per unit dry weight, was unaffected.

Increase in total leaf area closely paralleled that of total dry weight. It was slower in diseased plants and attained a lower limit than in healthy ones. From the 35th day after inoculation, onwards, diseased plants had fewer and smaller leaves and their leaves opened more slowly, one new leaf unfolding every 9 days on diseased plants against one every 4 days on healthy plants. Leaves of diseased plants were both smaller and thinner than those in corresponding positions on healthy plants.

The relationship between the absorbing system on the one hand, and the assimilating and transpiring system on the other, was greatly altered; the dry weight of fibrous root per unit leaf area was decreased after infection from 1·20 to 0·79 mg./cm.^{2} 20 days after inoculation and from 1·64 to 0·38 mg./cm.^{2} 82 days after inoculation.

Though infection consistently lowered net assimilation rate, on the average by about 15 per cent. of the control value, this decrease was never statistically significant.

Increases in the number of spores in the inoculum progressively decreased the dry weights of tops and fibrous roots, and sometimes increased the dry weight of clubs.

Delaying inoculation increased the initial growth rate of the galls, and sometimes their final size. The extent to which plants were damaged depended more on how long they had been infected than on age at inoculation.

INTRODUCTION

PLASMODIOPHORA BRASSICAE Woron., the clubroot pathogen, has a profound effect on the growth of its cruciferous hosts but no precise study of the changes seems to have been made. An opportunity for such a study arose in the course of experiments to test whether clubroot could be controlled by griseofulvin, a metabolite of *Penicillium* spp., with anti-fungal properties (Brian *et al.*, 1949). As effects of the antibiotic were being assessed

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by the weight of diseased tissue (clubs) produced and the weight of tops as representing yield, it seemed that the experiments might readily be extended to a quantitative analysis of how infection affects growth of the host plant. While clubroot is primarily due to the capacity of *P. brassicae* to cause the formation of a gall, some, perhaps important, symptoms may result from effects of the growing gall on other parts of the plant. These and other qualitative and quantitative relationships between various organs might be revealed by using the well-known methods of growth analysis (Gregory, 1921; Watson, 1952; Thomas, 1955) to compare healthy and infected plants. In addition, the value of arbitrary indices, used to assess severity of disease, might be clarified.

METHODS

Cabbage seeds of the variety 'King of Hearts' (Ryder & Sons Ltd., St. Albans, Herts.) were germinated on filter-paper supported above and dipping into tap water in glass-covered dishes, in the laboratory or in the greenhouse, at temperatures varying from 18° to 25° C. After 3–4 days the seedlings were transferred to a soil+sand mixture (pH 5·5—6·0) consisting of 1 vol. loam soil taken from old grassland and passed through a $\frac{1}{4}$-in. mesh sieve, plus 3 vol. silver sand, a medium suitable for clubroot experiments (Macfarlane, 1955). Earthenware flower-pots were used; when the percentage of plants diseased was to be determined 10 plants were grown in a 5-in. (rim diameter) pot, but for growth experiments it was usually more convenient to have one plant in a 3·5-in. pot. Several days were usually allowed to elapse between transplanting and inoculation, thus giving time for any replacements needed. The plants were grown in warmed greenhouses with a minimum temperature of *c.* 13° C., and the pots were arranged on the benches in ways designed to minimize position effects. No attempt was made to control soil moisture, but to ensure infection, the soil was kept thoroughly wet with rain water, with the pots allowed to drain freely. The following nutrient solution was applied at intervals of approximately 2 weeks:
$Ca(NO_3)_2.4H_2O$, 8·44 g.; KNO_3, 1·44 g.; $NaNo_3$, 1·85 g.; KH_2PO_4, 0·99 g.; $MgSO_4.7H_2O$, 3·52 g./l., 50 ml. to the 5-in. pots and 25 ml. to the smaller pots. Inocula consisted of resting spores of *Plasmodiophora brassicae* released from clubroot galls by maceration, washed in distilled water by centrifugation and stored as dense suspensions in distilled water at 4° C. (Macfarlane, 1958). The isolate of *P. brassicae* used had been maintained in the greenhouse on cabbage. To inoculate pots, known numbers of spores in distilled water or rain water were simply poured on to the soil surface.

Measurements were made of fresh weights and dry weights of tops and clubs, dry weights of fibrous roots (i.e. all healthy roots) and, in some experiments, leaf area. At harvest, tops were removed and weighed in the morning, when they were expected to be fully turgid, the stems being cut just below the insertion of the cotyledons and the hypocotyl left with the roots. When leaf areas were measured, all leaves completely or partly unfolded were stripped off the stem, arranged in order of age and shadow printed on 'blueprint' paper. Areas were measured later, mostly by planimeter or by cutting out the prints and weighing them. Petioles were included in the leaf area because they could not be sharply distinguished from laminae. Numbers of leaves and areas of leaves at known positions were obtained from the prints.

Roots were carefully washed free from soil over a fine sieve. Any part of the root system or hypocotyl that showed symptoms of clubroot was separated from the other roots as 'clubs', surface-dried and weighed. The recorded weights of young clubs may be slightly high, because of the impossibility of separating new gall tissue from previously existing tissue but the error incurred, like that caused by adding hypocotyls to fibrous roots, probably did not affect the main conclusions.

When the various parts of the plants had been separated, they were air-dried in a warm room for several days and then dried overnight at 80–90° C. before weighing. The results included both the actual measurements mentioned above and, when desirable, the following quantities derived from them, namely, water content per unit dry weight, and the various dry weights as appropriate percentages, and dry weight of fibrous roots per unit leaf area and the net assimilation rates (NAR). Net assimilation rates were calculated according to the usual formula:

$$\text{NAR} = \frac{(W_2 - W_1)(\log_e L_2 - \log_e L_1)}{(t_2 - t_1)(L_2 - L_1)},$$

where W_1 and W_2 are total dry weights and L_1 and L_2, leaf areas at times t_1 and t_2 respectively. As the sampling intervals were fairly short, errors caused by non-linearity of the relationship between dry weight and leaf area were presumably negligible (Williams, 1946). To get replicate values for NAR, rates were calculated for Expt. 1 in two ways. First, on the basis of a randomly selected pair of plants, one from each of two consecutive samples. In this way at least 8 values were obtained for each inter-sample period. Secondly, rates were calculated on the basis of 'block' means, giving two replicate values for each interval. In addition NARs were calculated from treatment means. For Expt. 2, in which there were fewer replicates but more treatments in factorial combination, rates were calculated from treatment means and the higher order interactions used as error (see below for details). Analyses of variance were done on the original figures when there was only one time of harvest. When there was a series of samples, the logarithms of the original figures were analysed, to avoid error-dependence on the mean. For a few measurements, where it seemed desirable to present original figures, errors were calculated for each sample separately (Figs. 1, 2, and 3). Percentages were first transformed to angles before being analysed, except for water contents as per cent. unit dry weight (Fig. 1) where it was unnecessary. NARs were analysed without transformation. The term 'least significant difference' is usually abbreviated to 'L.S.D.'.

Design and practical details of the experiments. Most of the experiments

TABLE I

Details of Experiments

Experiment	Date of planting 2–3 day-old seedlings	Size of pots (rim diameter)	Dates of inoculation	No. spores per inoculated pot	Dates when nutrients given	Dates of sampling	Total no. treatments per experiment	No. treatments relevant to present paper	No. replicates per treatment per sampling
1. Growth analysis 1955	23 Aug.	3·5 in.	27 Aug.	$1\cdot25 \times 10^{7}$	29 Aug.; 3, 17 Oct.	6, 16 Sept.; 1, 13, 17, 26 Oct.	4	2	8
2. Growth analysis 1956	5, 9 June	3·5 in. Then to 5-in. pots 19 July and to 7-in. pots 21 Aug.	8, 9 June	$2\cdot50 \times 10^{5}$, $1\cdot25 \times 10^{7}$	8, 9, 25 June; 5, 20 July; 10, 26 Aug. (double dose)	18, 28 June; 9, 18, 27 July; 9, 20 Aug.; 17 Sept.; 1 Oct.	18	2	2
3. Varying spore load 1955	3 Aug.	5 in.	5 Aug.	$4\cdot00 \times 10^{4}$, $2\cdot00 \times 10^{5}$, $1\cdot00 \times 10^{6}$, $5\cdot00 \times 10^{6}$, $2\cdot50 \times 10^{7}$	6, 28 Aug.	13, 14 Sept. (3 blocks); 23 Sept. (2 blocks)	30	6	5
4. Varying spore load 1956 (*a*)	17 Aug.	3·5 in.	6 Sept.	$1\cdot25 \times 10^{4}$, $4\cdot00 \times 10^{5}$, $1\cdot25 \times 10^{7}$	17 Aug.; 5, 18 Sept.; 1, 16 Oct.	18–22 Oct.	36	4	3
(*b*)	7, 8 Sept.	3·5 in.	20 Sept.		7, 18 Sept.; 1 Oct.	23–24 Oct.			
5. Varying time of inoculation 1955	3 Aug.	5 in.	5 or 19 Aug.	$2\cdot50 \times 10^{6}$, $2\cdot50 \times 10^{7}$	6, 28 Aug.; 16 Sept.	26–30 Sept.	54	3	4
6. Varying age of plant when inoculated 1956	3, 10, 17, 24 Aug.	3·5 in.	24 Aug.	$1\cdot30 \times 10^{7}$	17, 24, 30 Aug.; 13, 26 Sept.; 12 Oct.	19, 26 Sept.; 10, 16 Oct.	4	4	2

In experiments 3 and 5, ten plants were grown in each pot.

were designed to explore effects of the antibiotic griseofulvin on clubroot as well as effects of infection on cabbage growth. Series with griseofulvin treatment are seldom quoted in the results below, but were usually included in the analysis of variance. They are therefore mentioned briefly in the following description of the experiments. Practical details of the experiments are shown in Table 1.

Experiment 1 consisted of an uninoculated and an inoculated series, with and without griseofulvin, making a 2 × 2 factorial. Sample pots were removed at intervals.

Experiment 2 was similar except that there were 9 inoculation treatments including 2 spore loads and an uninoculated series. These were repeated with griseofulvin.

Experiment 3 tested 6 levels of inoculum, including no inoculum, in combination with 5 doses of griseofulvin, including zero dose.

Experiments 4*a* and 4*b* each consisted of an uninoculated series and three levels of inoculum. Nine griseofulvin treatments were combined with each.

Experiment 5 compared inoculation at planting with inoculation 2 weeks later. For each time of inoculation there was an uninoculated series and 2 series with different amounts of inoculum. These were combined with 9 griseofulvin treatments.

Experiment 6 compared plants of 4 different ages inoculated at one time.

RESULTS

Growth analysis of uninoculated and inoculated plants

The data collected in Expt. 1 enabled growth of the plants to be analysed in terms of the antecedent growth of roots and the assimilating system. The results from Expt. 2, given later in Fig. 4, varied considerably but generally confirm the trends shown by the more precise Expt. 1, and also indicate the rapid disorganization of the plant once the clubs rotted, 100 days after inoculation. The results of Expt. 1 will now be considered in detail.

Observations on infection. At the 10th day, zoosporangia of *P. brassicae* were seen in root hairs of inoculated plants and some had discharged their zoospores. By the 20th day, 8 of the 12 inoculated plants sampled showed unmistakable though very slight, swellings on the main root—first sign of developing clubroot galls. In spite of being thus recognized and the general principle adopted of treating any root recognizably invaded by *P. brassicae* as a club, the roots of these plants were regarded as 'healthy' because the swellings were so slight as to make the error caused by the impossibility of separating diseased from healthy tissue unjustifiably great. Thirty-five days after inoculation, at the 3rd sampling, sizeable clubs had formed on all inoculated plants.

Changes in dry weight and its distribution. Total dry weights of both control and inoculated plants continued to increase till the last sampling. Although clubs were formed by the 35th day, the total dry weight of healthy plants was not greater than that of the infected ones until the 60th day (Table 2). Thirty-five days after inoculation, infection had already decreased the dry weight of tops, which subsequently increased more slowly than in the controls. In this particular experiment the dry weight of total roots remained unaffected but the dry weight of fibrous roots was decreased by infection as soon as and more severely than that of tops, and thereafter hardly increased further—the difference between the weights of total and fibrous roots being the weights of the progressively increasing clubs.

TABLE 2

Effect of Inoculation with P. brassicae *on the Dry Weight of Cabbage (Expt.* 1)

Each figure is the mean of at least 8 replicates and least significant differences were calculated on this basis

(For details see Methods, p. 551 and Table 1)

Part of plant	Experimental condition	Means of logarithms of dry wts. (mg.) Days after inoculation						Least significant difference ($P = 0·05$)
		10	20	35	47	60	82	
Total	Control	1·39	2·04	2·94	3·33	3·54	3·78	0·18
	Inoculated	1·43	2·14	2·83	3·18	3·32	3·45	
Tops	Control	1·23	2·00	2·81	3·18	3·40	3·63	0·12
	Inoculated	1·25	2·03	2·65	2·87	2·98	3·06	
Total roots	Control	0·84	1·59	2·37	2·81	2·93	3·16	0·17
	Inoculated	0·95	1·50	2·35	2·88	3·04	3·16	
Fibrous roots	Control	0·84	1·59	2·37	2·81	2·93	3·16	0·20
	Inoculated	0·95	1·50	1·93	2·00	2·14	2·04	
Clubs	Inoculated	—	—	2·11	2·82	2·98	3·12	0·21

The various dry-weight ratios given in Table 3*a* demonstrate the changes in distribution of dry matter resulting from infection. In healthy plants there was no change in the proportion of the total dry weight in the roots during the experiment. In infected plants the proportion in the roots was significantly greater at the 35th day and increased till, at the final sampling, about half the dry weight of the plant was in the roots. At the 35th day clubs already constituted about a quarter of the total dry weight and half the root weight, while at the 82nd day clubs formed somewhat less than half the total dry weight and three-quarters of the root weight. These measurements show clearly enough the main effects of infection; clubs developed at the expense of tops and of fibrous roots, especially the latter. Further, the dry-weight ratios also indicate a relative decrease in roots of infected plants at the 20th day. At the 20th day the proportion of total dry weight in the roots of infected plants was lower, though not quite significantly so, than that in the roots of healthy plants, but was significantly lower than the proportion in roots of infected plants at the 10th day. This last value was rather high, being greater than that for the control plants, and this may have been fortuitous. The same result is emphasized more when dry weights of roots are considered in relation to unit leaf area (Table 3*f*). At the 20th day there was a very considerable drop in this ratio. If infection caused a temporary check to root growth, movement of materials down to the root might be slower thus

TABLE 3

Effect of Inoculating Cabbage with P. brassicae *on:* (a) *Relative Distribution of Dry Weight,* (b) *Total Leaf Area,* (c) *Mean Leaf Area per leaf,* (d) *Respective Contribution of Number and Size of Leaves of Total Leaf Area,* (e) *Fresh Weight of Unit Area of Leaf,* (f) *Dry Weight of Fibrous Root per Unit Leaf Area. (Expt. 1)*

(Means of at least 8 values; least significant differences computed on basis of this number of replicates. (*b*) Total leaf area: all living leaves including a few < 1 cm.² (*c*) Mean leaf area per leaf: total area of all leaves of 1 cm.² or more ÷ corresponding number of leaves. For details see Methods, p. 551 and Table 1)

Characteristic		Experimental condition	Days after inoculation 10	20	35	47	60	82	Least significant difference ($P = 0·05$)
(*a*) Means of angular values of dry wt. ratios expressed as percentages	$\frac{\textit{Total roots}}{\text{Total plant}}$	Control	32·9	32·2	31·2	33·2	30·1	30·3	4·10
		Inoculated	35·7	28·6	35·4	45·6	47·0	48·4	
	$\frac{\textit{Clubs}}{\text{Total plant}}$	Inoculated	—	—	26·7	41·7	42·9	45·7	5·20
	$\frac{\textit{Clubs}}{\text{Total roots}}$	Inoculated	—	—	50·5	68·8	68·9	73·3	5·90
(*b*) Means of logarithms of leaf area per plant (cm.²)		Control	0·86	1·54	2·25	2·57	2·77	2·93	0·12
		Inoculated	0·84	1·56	2·12	2·34	2·43	2·53	
(*c*) Means of logarithms of cm.² of mean leaf area per leaf of 1 cm.² or more		Control	0·31	0·82	1·28	1·56	1·65	1·70	0·10
		Inoculated	0·31	0·83	1·18	1·45	1·49	1·52	
(*d*) Actual values for inoculated plants expressed as percentages of controls	Leaf area per plant	—	99	104	73	61	46	41	—
	No. leaves present	—	100	104	94	78	67	67	
	Mean area per leaf	—	99	101	83	74	67	65	
(*e*) Fresh wt. of unit area of leaf (mg. per cm.²)		Control	33·9	35·7	35·1	46·3	44·5	52·8	2·90
		Inoculated	34·7	34·2	35·2	39·0	37·1	38·3	
(*f*) Dry wt. fibrous root per unit leaf area (mg. per cm.²)		Control	1·16	1·20	1·35	1·77	1·48	1·74	0·36
		Inoculated	1·33	0·79	0·65	0·47	0·55	0·38	

making more available for top growth. Examination of the original data showed that there was a small decrease in the weight of roots and a small increase in the weight of tops and in leaf area, although means of logarithms of these figures showed no significant differences (Table 2). The differences in actual dry weights and areas, however, were evidently sufficiently great for ratios calculated from them to show significant differences. This effect of infection on dry-weight ratio was not found in Expt. 2 possibly because the results were so variable.

Water content. Infection had no effect on the succulence of the tops as measured by water content as a percentage of dry weight (Fig. 1). On this basis water content diminished considerably between the 10th and 35th day, as might be expected because of increased laying down of woody and other structural elements, and then remained fairly constant. Measurements for leaves alone, without stems, were available for two of the sampling dates. At the 47th day the mean percentage value for the tops was 1,059, that for leaves of healthy plants 1,081 and that for leaves of infected plants 1,062. The corresponding figures at the 60th day were 1,049, 1,093 and 1,050 respectively. Water contents of leaves, at these times at least, corresponded closely to those of entire tops.

Water contents of clubs, measured in the same way, were much lower than those of leaves and declined with increasing age of the galls, probably as they accumulated starch.

Changes in the leaves. Changes in the dry weight of tops caused by infection were closely paralleled by changes in leaf area (Table 3*b*). At the 35th day leaf area of infected plants was just significantly lower than that of healthy plants and thereafter the difference increased. Numbers of leaves present and the mean area per leaf of infected plants were both lower than those of healthy plants by the 35th day and the difference widened with each successive sampling (Table 3*c*). Table 3*d* shows these quantities for infected plants, expressed as percentages of the respective values for control plants, and shows that the decrease in number of leaves and the decrease in their mean area contributed about equally to the loss of total leaf area.

Few leaves smaller than 1 cm.^{2} were recorded and their omission made little difference to the results. Very few leaves died during the course of the experiment, apart from the cotyledons and a few leaves in the first position. Any of the latter, although not the cotyledons, are taken into account in Fig. 2 which shows the number of leaves greater than 1 cm.^{2} in area produced by healthy and infected plants up to each sampling time. The curve for the control plants was obviously linear, and a straight line was fitted. The rate of leaf formation was diminished after clubs formed and a separate straight line was fitted to the diverging limb of the curve for infected plants. From the equations of these lines it was found that a new leaf was unfolded every 8·7 days in healthy plants and in 4 days for plants infected 35 days or more. How much these rates diverged from true rates of leaf initiation at the apex is not known, as the number of leaves in the bud was not determined.

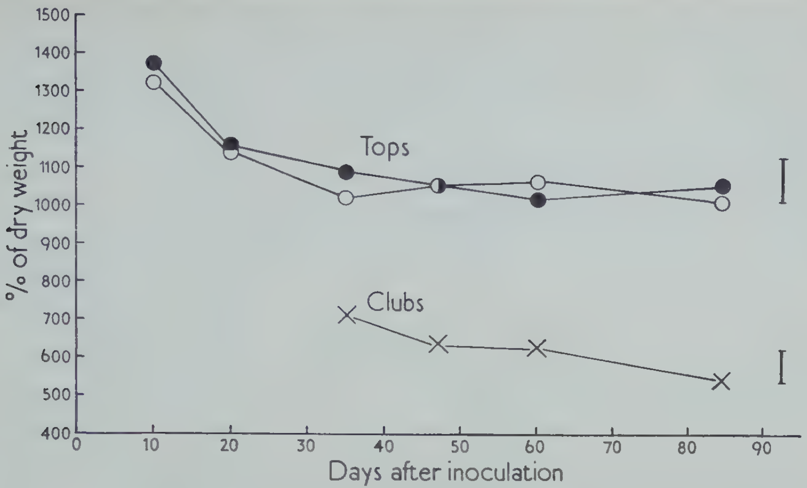

FIG. 1. Succulence of control and inoculated cabbage plants (Expt. 1)
Water contents expressed as percentages of dry weights. ○, tops of control plants; ●, tops and ×, clubs of inoculated plants. Means of at least 8 replicates. Least significant difference ($P = 0{\cdot}05$) indicated on the right.

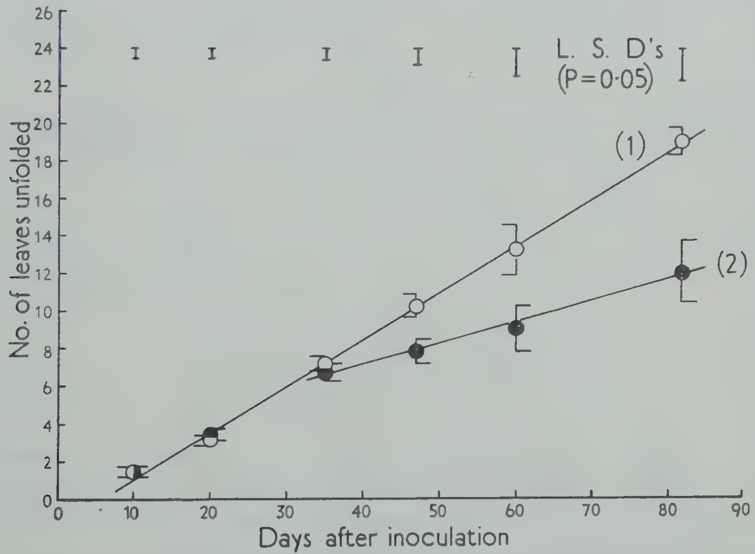

FIG. 2. Effect of inoculation with *P. brassicae* on the number of leaves greater than 1 cm.^{2} produced by cabbage (Expt. 1).
○, control; ●, inoculated. Means of at least 8 replicates.
5 per cent. fiducial limits are indicated: ○ or ●.
Equations: (1) $y = 9{\cdot}095+0{\cdot}2476\,(x-42{\cdot}33)$
(2) $y = 8{\cdot}995+0{\cdot}1146\,(x-56{\cdot}00)$

Cabbage 'heads' were not formed by any plants, but the apical bud obviously increased in size particularly in the controls. Numbers of leaves initiated would therefore be progressively underestimated as the plants grew older, and this discrepancy was probably greater for the healthy than for the infected plants.

Fig. 3 shows growth curves for the first 9 leaves, numbered from the first leaf above the cotyledons, of healthy and infected plants. Least significant differences were calculated only for the 3rd and 6th leaves, merely to give some idea of the magnitude of the error. Infection clearly affected the 3rd leaf which had already unfolded by the 20th day. The effect of infection in decreasing leaf size was progressively greater with successively younger

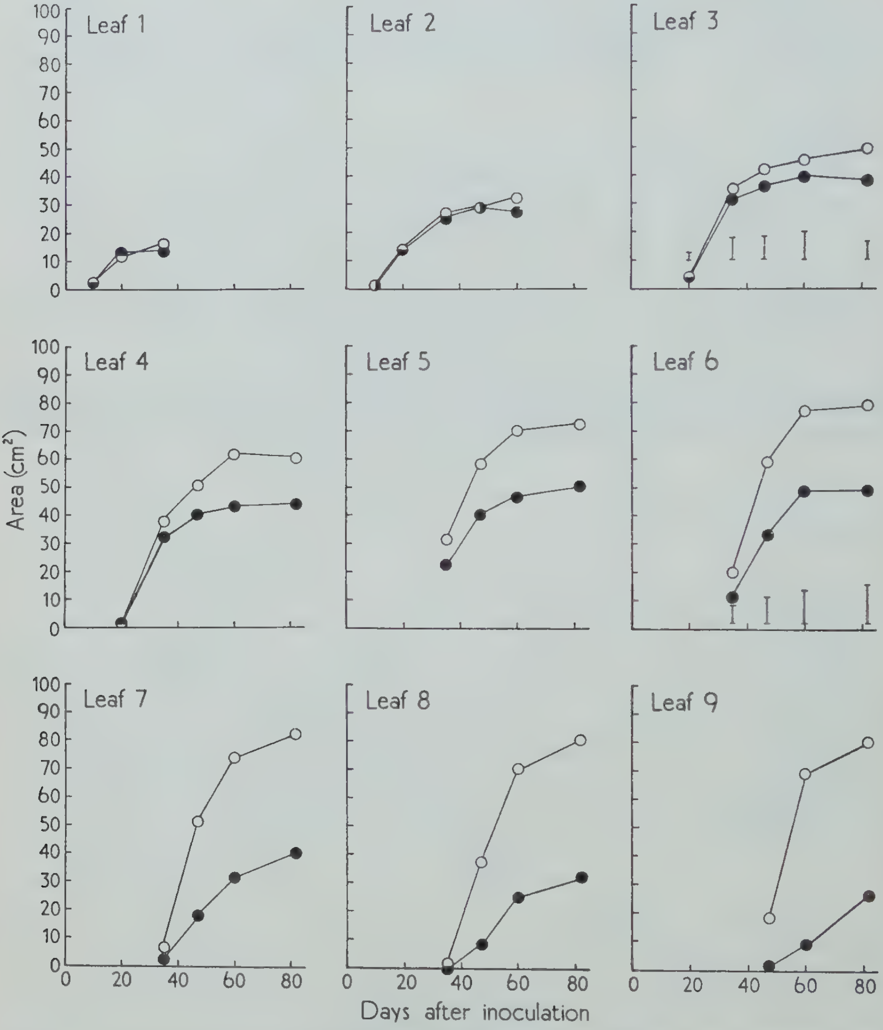

FIG. 3. Growth in area of individual leaves of cabbage, un-inoculated and inoculated with *P. brassicae* (Expt. 1).

○, un-inoculated; ●, inoculated. Leaf 1 = 1st leaf above cotyledons. Least significant differences (P = 0·05) given only for leaves 3 and 6.

leaves. This was probably due to the increasing effect on cell division in the leaf primordium caused by the drain of materials to the gall, in addition to effects of infection on the expansion of the leaf.

Fresh weight of unit leaf area of control plants increased particularly from the 35th day onwards; those of infected plants showed only a slight increase (Table 3*c*). Because water contents per unit dry weight were similar in control and infected plants and did not change after the 35th day, the mean leaf thickness in control but not in infected plants must have steadily increased—so confirming the impression of greater thickness in the controls as given by appearance and touch.

Dry weights of fibrous root per unit leaf area (Table 3*f*) were computed to relate the water and nutrient absorbing system to the water-losing and assimilating system. From the 35th day the amount of roots of control plants tended to increase in relation to leaf area, although it varied considerably, whereas for infected plants this quantity decreased almost throughout the experiment. Its decline even at the 20th day has already been noted. From the 47th day onwards there was about one-quarter the amount of fibrous root per unit leaf area in the diseased compared with the healthy plants. These figures strikingly demonstrate the effect of disease in decreasing the potential capacity of the plant to make good its water loss and maintain its supply of mineral nutrients.

TABLE 4

Net Assimilation Rates of Control and Inoculated Plants (Expt. 1)

(mg. per $cm.^2$ per wk.)

Calculated from:	Experimental condition	Sampling intervals: days after inoculation					Least significant difference ($P = 0·05$)	No. replicate determinations of NAR treatment
		10–20	20–35	35–47	47–60	60–82		
'Random pairs of single plants', one from each of 2 consecutive samples	Control	5·50	3·35	2·90	1·44	1·03	0·86	8
	Inoculated	4·70	2·84	2·38	1·19	0·82		
	Mean	5·10	3·10	2·64	1·31	0·93	0·61	—
'Block' means	Control	4·67	4·08	3·00	1·41	1·05	1·12	2
	Inoculated	4·17	3·37	2·66	1·22	0·73		
	Mean	4·42	3·73	2·83	1·32	0·89	0·78	—
'Treatment' means	Control	4·65	3·93	2·77	1·41	1·05	—	1
	Inoculated	4·28	3·44	2·57	1·24	0·72		
	Mean	4·47	3·69	2·67	1·33	0·89	—	—

Net assimilation rates. Net assimilation rates, of both control and infected plants, declined throughout the experiment irrespective of the method of calculation (Table 4). Rates for infected plants were consistently slightly lower, even for the first inter-sample period, but at no time did the difference between control and infected series reach the significance level, nor was the overall effect significant.

Effect of number of spores in the inoculum on the changes in growth caused by infection

Inoculation significantly decreased fresh weight of tops, but the progressive decline with increasing numbers of spores was not quite statistically significant (Table 5). Varying the amount of inoculum had little effect on fresh weight of clubs. Total dry weight decreased for each successive increase in spore load but was not significantly less than the control value until the spore load was increased to 10^6 per pot. With the maximum amount of inoculum applied, total dry weight was significantly lower than with the smallest. Similarly, the dry weight of tops was decreased by infection and at the two highest spore loads was significantly lower than at the lowest. There was, as with the fresh weights, an almost continuous gradation of dry weight with increasing inoculum. Dry weight of fibrous roots was greatly decreased by infection. Weight of these roots also decreased with increasing spore load, but differences were not significant. Dry weight of clubs was scarcely affected except that, at the highest inoculum level, it was lower probably because of earlier secondary rotting.

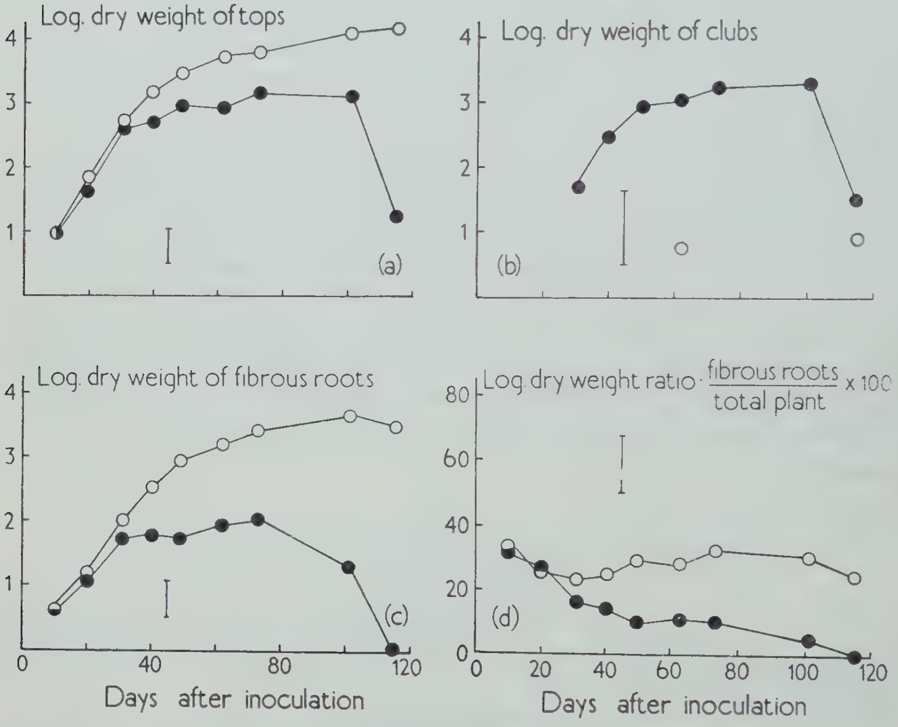

FIG. 4. Summary of effects of inoculating cabbage with *P. brassicae* (Expt. 2). ○, control; ●, inoculated. Means of values for 2 treatments with different spore loads, each with 2 replicates. The values in figs. *a*, *b*, *c* are means of log. dry weight (mg.); in (*d*) they are means of angular transformations of the percentages.

The effects of varying the number of spores were more evident in Expts. 4*a* and 4*b* (Table 6). In Expt. 4*a* total dry weight was just significantly decreased by the lowest spore load, only the highest spore load giving a further significant decrease. Each addition of inoculum decreased dry weights of tops and of fibrous roots and increased the weight of clubs. Weight of total

TABLE 5

Effect of Number of Spores in the Inoculum on Fresh Weight and Dry Weight of Cabbage (Expt. 3)

(Each figure is the mean of 5 replicates. For details see Methods, p. 548 and Table 1)

		Means of fresh wts (g.) of:		Means of dry wts. (g.) of:			
		Tops	Clubs	Total Plant	Tops	Fibrous roots	Clubs
No. spores per pot	None	48·9	0·0	4·77	3·81	0·96	0·00
	4×10^{4}	35·8	11·2	4·53	2·84	0·37	1·33
	2×10^{5}	32·8	11·5	3·90	2·30	0·27	1·32
	1×10^{6}	29·6	11·0	3·63	2·13	0·22	1·28
	5×10^{6}	25·2	11·2	3·37	1·82	0·20	1·34
	$2·5\times10^{7}$	26·3	10·3	3·18	1·84	0·16	1·18
L.S.D. ($P = 0·05$)		7·9	1·1	1·07	0·76	0·26	0·35

TABLE 6

Effect of Number of Spores in the Inoculum on Dry Weight of Cabbage (Expt. 4)

(Each figure is the mean of 27 values from all treatments, including those with griseofulvin, in Expt. 4. For details see Methods, p. 548 and Table 1)

		Means of dry wts. (cg.) of:					Means of dry wt. ratios: Clubs as per cent. of total plant
		Total plant	Tops	Total root	Fibrous root	Clubs	
Expt. *4a*	None	442	344	98	97	1	0·2
No. spores per pot	$12·5\times10^{3}$	412	307	105	71	33	8·1
	$4·0\times10^{5}$	413	278	135	48	87	21·1
	$12·5\times10^{6}$	392	226	166	28	138	34·9
L.S.D. ($P = 0·05$)		29	23	15	9	17	3·6
($P = 0·01$)		39	31	20	12	22	4·7
Expt. *4b*	None	187	153	34	21	13	7·9
No. spores per pot	$12·5\times10^{3}$	174	127	47	10	37	21·3
	$4·0\times10^{5}$	154	100	53	6	47	31·2
	$12·5\times10^{6}$	138	85	53	5	48	34·6
L.S.D. ($P = 0·05$)		15	14	8	3	8	5·3
($P = 0·01$)		20	18	10	4	11	7·0

roots was increased by inoculation with the fewest spores and significantly increased by further amounts of inoculum. Similarly, the percentage, in the clubs, of the total dry weight was increased with each increase of inoculum. The small weight of clubs in the controls was due to a few contaminations. Expt. *4b* was set up slightly later than *4a*, towards the end of the growing season, and was harvested sooner after planting and inoculating. The dry-weight yield was consequently lower and the effects of increasing numbers of spores did not exactly duplicate those obtained in Expt. *4a*. The lowest level of inoculum did not quite significantly decrease total dry weight, although each successive increase in inoculum did. Once again each addition of inoculum significantly decreased dry weight of tops. Infection increased dry weight of total roots above that of controls, but spore load had no significant effect. Weight of fibrous roots was decreased except by the last addition of inoculum. Similarly club weight and the proportion, in the clubs, of total dry weight were not affected by the increase in spore load from the intermediate to the highest level, but were increased by the difference from low to intermediate levels. At the lowest concentration the proportion of the total dry weight in the clubs was considerably higher than with the same treatment in Expt. 4*a*, but at the maximum spore load the proportions were about the same in both experiments, *c.* 35 per cent.

Effect of varying the age of the plant when inoculated on its subsequent growth

In Expt. 5 (Table 7) inoculation 2 weeks after planting decreased the dry weights of the total plant, tops and fibrous roots less than inoculation at planting; it also diverted less of the total dry weight to clubs although the clubs were larger. Clearly, delaying inoculation enabled the plant to make more growth before there was any interference by the developing gall. The larger productive unit might then even support a larger club.

TABLE 7

Effect of Varying the Time of Inoculation on Dry Weight of Cabbage (Expt. 5)

(Each figure is the mean of 8 replicates, 4 from each of two series with different amounts of inoculum. For details see Methods, p. 548 and Table 1)

		Means of dry wts. (g.) of:				Means of dry wt. ratios: Clubs as per cent. of total plant
		Total plant	Tops	Fibrous roots	Clubs	
Inoculation	None	6·55	5·16	1·30	0·00	0·0
	At planting	3·67	2·04	0·22	1·42	37·9
	2 weeks after planting	5·21	3·24	0·40	1·54	29·6
L.S.D. (P = 0·05)		0·58	0·41	0·16	0·23	3·4

Effects of this kind are shown better by the results of Expt. 6, in which plants of four ages differing by 1 week, the youngest newly germinated, were inoculated at the same time and samples were taken at four subsequent dates. There were also uninoculated plants for each age-group. The arrangement of this experiment allows first, a comparison of plants of the same age at harvest but infected for different periods as in Expt. 5, and secondly, a comparison of plants of different age infected for a similar period before harvest.

Comparison of plants of the same age at harvest showed that, as in Expt. 5, delaying inoculation increased the yield of tops and fibrous roots although the clubs also grew larger.

The effects, measured at the same harvest, of varying age of the plant when inoculated appeared more interesting and less obviously expected. By plotting the differences between the logarithms of dry weights of infected plants and the corresponding uninfected plants against age at inoculation, it could be determined whether plants inoculated at different ages were differently damaged. These data, given in Fig. 5, are the means of the 4 sampling times and, being logarithmic, represent proportional differences in actual dry weight. On this basis, there was little effect of age at the time of inoculation, except that the 2 week-old plants tended to be rather more severely damaged than the others. This is seen most clearly in the differences in fibrous roots, where the greatest divergence from control values occurred. The decrease in weight of fibrous roots caused by inoculating when 2 weeks old was significantly greater than when 0, or 1 week old, and almost significantly greater than when 3 weeks old. The apparent initial growth-rate of clubs, measured by actual dry weight or logarithms of dry weight (Fig. 6), was increased strikingly by increasing the age of the plant when it was inoculated. This was to some extent inevitable, as club weight was zero at the time of inoculation and, in the older and larger plants, more previously uninfected tissue must have been included in the 'clubs' (see Methods, p. 548). Also, this tissue would probably be more lignified in the older plants and so have a relatively greater dry weight. It seems unlikely, however, that this unavoidable error in measuring clubs could alone account for the hundredfold difference between the values for the oldest and youngest series. In the corresponding controls, weights of total roots differed by a factor of just over ten. How much root weight of the controls increased during the infection period is not known, as root weights at the time of inoculation were not measured. After the first harvest the rates of increments of the logarithms of the dry weights were roughly similar.

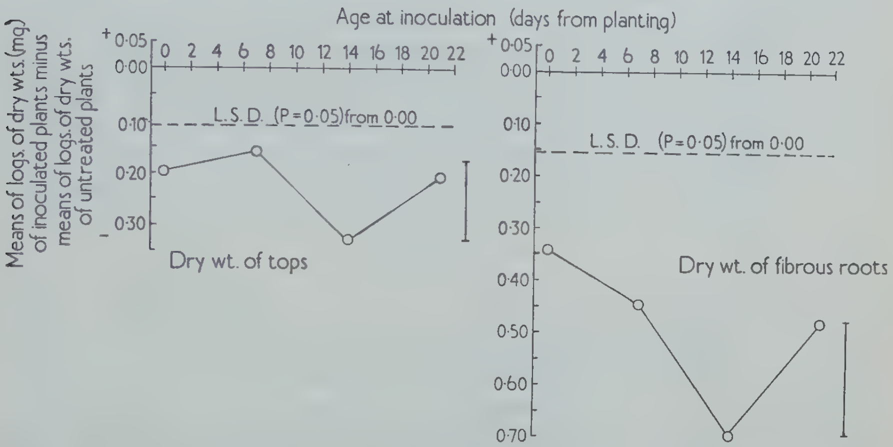

FIG. 5. Assessment of damage caused by inoculating cabbage plants of different age with *P. brassicae* (Expt. 6), negative values obtained by subtracting logarithms of dry weights (in mg.) of un-inoculated plants from similar values for inoculated plants.

Each point based on 8 replicates, 2 from each of 4 sampling times.

The short vertical lines give the least significant differences between points ($P = 0{\cdot}05$).

Although, at the first harvest, the weights of clubs were considerably greater on the older than on the younger plants, they constituted only a slightly greater proportion of the total dry weight (Fig. 7). Thereafter the

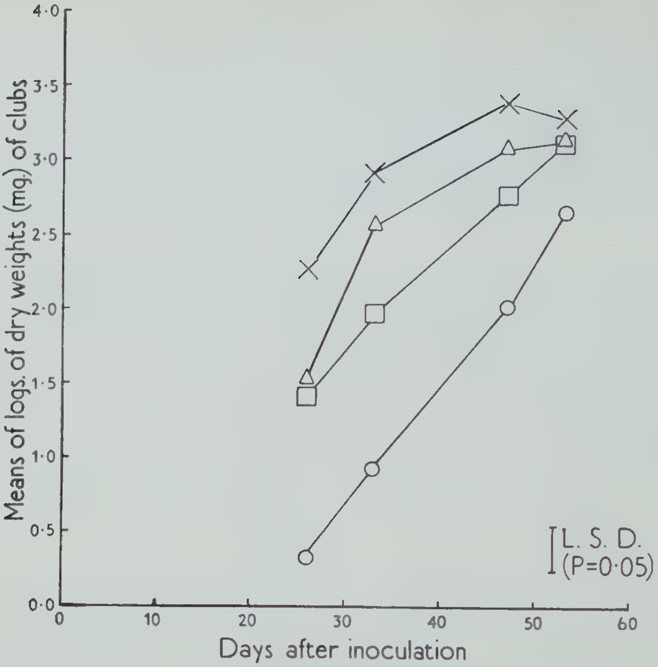

FIG. 6. Effect of age of plant when inoculated with *P. brassicae* on the subsequent growth of clubs (Expt. 6).

Cabbage seedlings planted when 2 days old.

Inoculated: ○, at planting; □, after 7 days; △, after 14 days; ×, after 21 days. Means of 2 replicates.

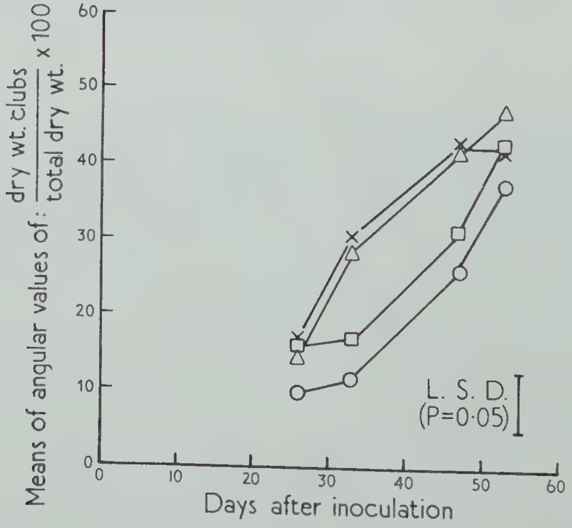

FIG. 7. Effect of age of plant when inoculated with *P. brassicae* on changes in distribution of dry weight (Expt. 6).

$$\frac{\text{Dry wt. clubs}}{\text{Total dry wt.}} \times 100$$

Cabbage seedlings planted when 2 days old. Inoculated: ○, at planting; □, after 7 days. △, after 14 days; ×, after 21 days. Means of 2 replicates.

proportion, in the clubs, of total dry weight increased in all series, but, at first, more slowly in those inoculated at planting or when 7 days old than in the others. The proportion, in the total root system, of the total dry weight of the plant tended to follow a similar pattern, except for the greater proportion of dry weight in the roots of the 21-day series at the first sampling. In the controls differences in the proportion of total dry weight of the plant present in the roots, either at different sampling times or with different planting dates, were negligible. The proportion of the dry weight of the total root present in the clubs tended to be lowest in the plants inoculated at planting until the final sampling, when it approached the values for the others.

It was also noted that control plants of the same age at harvest weighed more when planted early than when planted late. This was probably due to seasonal or weather changes in temperature, light intensity, &c.

DISCUSSION

While the formation and growth of the clubroot gall are, of course, inseparable from effects the gall causes on the remainder of the host plant, it will be convenient to discuss the results in relation to those two aspects of the disease, namely, (1) the growth of the parasite and its immediate effect on the host of forming the gall, (2) consequent alterations in host metabolism.

Growth of Plasmodiophora brassicae *and initiation of the gall.* The stage of *P. brassicae* associated with clubroot galls was first described by Woronin (1878) and is sufficiently well-known to need no further comment here. Precisely how and when this stage originates is uncertain, but it is thought likely that it is initiated by the products of discharge of the zoosporangia that occur in root-hairs as a result of primary infections from resting spores (Bremer, 1954; Macfarlane, 1955). The suggestion in Expt. 1, 20 days after inoculation, that *P. brassicae* affected root growth soon after zoosporangia had discharged and by the time that galls were only just beginning to form, is therefore of particular interest. Discharge of the zoosporangia had in fact begun 10 days after inoculation and in the following 10 days there was a sixfold increase in the weight of roots. A change caused by *P. brassicae* may therefore have had an appreciable effect by the 20th day. Even though clubs had been initiated, the proportion of dry weight in the roots tended to be lower than in uninfected plants, rather than higher, as it is in plants with well-formed clubs. Perhaps at the early stages of invasion of the main root, the development of existing roots and root initials was retarded by an inhibitor produced by *P. brassicae.* The decline in growth would then account for decreased movement of dry matter to the roots, more being retained in the tops. Such an inhibition would, of course, have to be temporary or sufficiently incomplete to allow the later limited growth of fibrous roots.

These effects seem comparable to those reported by J. E. Smith (1955) on the effects of auxin and other substances on the growth of excised infected roots. Smith suggested that in the initial stages of club formation, or perhaps even when the parasite was in the root-hairs, infection resulted in internal concentrations of auxins supra-optimal for the growth of root tips. This recalls the mutual interactions of leguminous root nodules with other nodules and lateral roots, particularly the effect of ineffective nodules on formation of laterals (Nutman, 1952) but, in the absence of similar experiments on club-root, the analogy should perhaps not be unduly stressed.

As the importance of growth substances in the development of crown gall, some insect galls, and of various types of tissue culture is now well established, changes in the metabolism of growth substances in the cabbage, when clubs are formed, are virtually certain. The nature of the stimulus to club formation, however, has not been thoroughly investigated. Hawker (1950) reported some limited experiments in which extracts of clubs cause swellings on cabbage seedling roots similar to those caused by suitable concentrations of indole-3-acetic acid. An effect on cell division in advance of the pathogen was noted by Kunkel (1918) and ascribed, in effect, to a growth-stimulating substance, but there is no evidence that appreciable effects occur at any great distance, and the growth of clubs seems very closely associated with the presence of the parasites. Thus, increasing numbers of spores in the inoculum increased the rate of gall growth and sometimes the final size of the gall, which was also affected by the age (or size) of the plant at inoculation. Inoculation of a large plant may result in more rapid growth of the club, initially at least, as in Expt. 6, than inoculation of a small plant. Somewhat similar results were reported by Katterfeld (1923). At first sight this result appears due simply to the greater capacity for growth of the larger plant. Although this probably accounts, at least in part, for differences in absolute size of the galls, other factors may be important. The more extensive root system of the larger plant might have been invaded at more points than that of the smaller plant and a greater bulk of tissue invaded. Again, the ratio of parasites to susceptible tissue might have increased and this might have provided a greater stimulus to gall formation. The graphs of logarithms of dry weights against time suggest that the relative growth-rates of galls on both young and old plants were similar, so differences in their final size might have depended on the amount of tissue invaded when the club started to grow. It is also possible that *P. brassicae* develops more rapidly in cambial than in other types of tissue. In a very young plant it may take some time before root cambium is formed and growth of *P. brassicae* may be initially slow and the formation of the gall delayed. Such an explanation would account for the lag in growth of clubs on young plants. However, galls can develop where there is no cambium, such as the cortical galls described by Kunkel (1918) and Larson (1934), although wounding is usually necessary and this may stimulate cell division. Also, plasmodia may migrate, presumably in the cambium, up the stem to the apex and invade and distort the young leaves, without causing a typical gall (Walker, 1942).

As it is implied in the foregoing discussion that growth of the galls and multiplication of *P. brassicae* proceed simultaneously, it follows that the growth of *P. brassicae* can be assessed by the growth of the gall. The usefulness of such a measurement must depend on a constant proportion of the dry weight of the gall being associated with thalli of *P. brassicae.* Kunkel (1918) found that, although the proportion of infected cells in a club varied considerably from plant to plant, *P. brassicae* consistently occupied about 28 per cent. of the volume of the gall. To what extent this relationship varies according to changes in the environment is not known. With this possible limitation in mind, and in the absence of an efficient extraction to enable the total number or weight of spores produced to be separated and measured, the weight of the gall can be regarded as an approximate guide for comparative purposes.

Alterations in the host plant. The principal effect of clubroot disease on the cabbage plant is clearly to alter the distribution of dry matter. Materials are diverted to the developing gall at the expense of shoots and fibrous roots. Secondary effects follow with the diminishing increases in capacity for assimilation and absorption.

These effects caused by developing clubroot galls can also be regarded partly, if not entirely, as the expected consequences of a new, and increasing meristem activity, arising within the plant. Broadly similar sequestration of material by intensely active meristems such as crown galls, where close similarities to clubroot might be expected (Levine, 1921), naturally occurring buds, and chemically induced growths, have been observed. Thus Eaton (1931) found that flower buds and even vegetative buds of the cotton plant developed at the expense of existing roots and leaves. When both were removed transport of carbohydrates to and accumulation of nitrogen in the roots were greatly increased (Eaton and Joham, 1944; see also Nutman, 1933). In the cotton plant a similar dominance in demand by developing fruits was found by Anderson and Kerr (1943) to extend to water. Referring to root-inducing hormones, Mitchell and Marth (1950) noted that 'It has been a general observation that some kind of cuttings can be overstimulated in root production, thereby utilizing a high percentage of their food reserves. This factor in combination with direct bud-inhibiting effects of the root-promoting substance may account for reduced top growth that is sometimes found with growth-regulator-treated cuttings' (see also Stuart, 1938). The direction in which nutrients move in a plant would seem to be greatly influenced by the position and number of dividing cells, and perhaps also their rate of division.

As soon as clubs appeared, increments in growth of tops and fibrous roots were decreased. The latter were affected considerably more than the tops, e.g. in Expt. 1 there was hardly any increase in fibrous roots of diseased plants after clubs had formed. This may have happened because the gall was between the tops and the fibrous roots, and thus where downward-moving material could readily be intercepted. Comparison with the observations of Levine (1921) on sugar-beet with crown galls is interesting. Total fresh weight of his plants was not significantly affected by infection with

Agrobacterium fumefaciens (E. F. Smith and Towns) Conn., but the weight of tops decreased considerably and the weight of roots rather less. The root decrease may have been incompletely recorded, as Levine's plants were grown in the field and many fibrous roots must have been lost. But it is likely that the roots suffered less because of the lateral position of the gall on the storage root so that material might not have been diverted from the roots as easily as in our clubroot-diseased plants in which the entire upper part of the main root became part of the gall.

Until the clubs rotted, the tops of diseased plants supported as large or even a larger total weight of root than did the tops of healthy plants, but inevitably failed to maintain their own relative increase. The marked decrease in the amount of fibrous (absorbing) root per unit of leaf surface in diseased plants shows how much the supply of nutrients and water to the tops may have been limited. How much of the root system of a healthy plant is necessary to maintain growth is not known, but the amount of unnecessary root probably varies a great deal with the species of plant and the prevailing environmental conditions. For instance, Weaver and Zinc (1946) reported that 'removal of half or even more of the root system had little harmful effect upon the growth of several species of grasses' and Went (1943) suggested that less than 10 per cent. of the root system was responsible for more than 50 per cent. of the growth-rate of the tops of certain experimental tomato plants. By contrast, Aldrich and Work (1934) found that much smaller changes in the ratio of root to leaf surface affected internal water deficit and the rate at which pears enlarge.

The slower rate of leaf production in diseased plants probably resulted largely from a decreased supply of food to the growing-point of the shoot. Smaller leaves would also result from restricted food-supply, as Eaton (1931) found in his dis-budding experiments on cotton. It could also be due to lack of nutrients from the roots (cf. small leaves in nitrogen-deficient plants (Morton and Watson, 1948)) although plants were well supplied with mineral nutrient solution and never showed signs of deficiency. Occasionally a slight interveinal mottling appeared when clubs were well developed, perhaps indicating a nutrient deficiency, but it could also be a sign of toxic effects of the pathogen or associated secondary organisms in older parts of the gall. There are reports that leaves of clubroot-diseased cabbage have a lower potassium content than leaves of healthy plants (Reed, 1911; Nicoloff and Stefanova, 1922), but it seems doubtful, with no signs of deficiency, whether this would appreciably affect carbohydrate metabolism by increasing respiration rate and lowering the rate of assimilation (Gregory and Richards, 1929).

The frequent wilting observed indicates that the water-supply to the tops of diseased plants was inadequate. Restricted water-supply to the shoot would be expected to diminish leaf expansion, as Thut and Loomis (1944) found for a range of plants. Lack of water might indicate not only insufficient absorbing root, but also impeded movement of water through the gall. There is no information for clubroot on this question and the closest parallel is the observation of Melhus *et al.* (1925) that the rate of movement of water through crown galls on apple shoots was decreased by 30 per cent. compared with that in a healthy shoot. Further, the disruption of the vascular system in clubroot-diseased plants would also affect transport in the phloem and in this way also help to limit growth of roots.

The water relationships of the diseased plant are not at all clear. That disease had no effect on succulence of the shoot is at first sight surprising. Fresh weights were, of course, determined as early in the morning as practicable and the diseased plants were then usually turgid. Even if water-supply were restricted, however, as seemed obvious, succulence need not have been affected, as Simonis (1952) has shown. Frequent wilting might be expected to cause xeromorphic tendencies (Tumanov, 1927), but there was no sign of these and there was evidence that the leaves of diseased plants were thinner than those of healthy plants. Drought hardening is usually associated with the accumulation of carbohydrates in the leaf (Vassiliev and Vassiliev, 1936; Levitt, 1956) and the lack of carbohydrates in the infected plants, principally because of their moving to the gall, may have tended to produce a shade type of leaf. If this were so then wilting might be attributed to an exceptionally high rate of water loss from leaves as well as to a limited water-supply. Wilting, whatever its cause, indicates an internal water deficiency, which can (Rabinowitch 1945; Loustalot, 1945) diminish the rate of photosynthesis. This would probably be reflected in the net assimilation rate and Morton and Watson (1948) reported that, with a restricted water-supply, NAR of sugar-beet was consistently lower than when water was given freely, the total decrease being about 15 per cent. of the higher value. Further, by analogy with other diseases (Allen, 1953), the respiration of infected tissues might be expected to exceed those of healthy tissues. If clubs were regarded only as excess meristematic tissue, then, because meristems respire more rapidly than mature tissue (Kidd, West, and Briggs, 1921), the respiratory losses of infected plants would exceed those of healthy plants. All these factors would tend to diminish NAR and, in Expt. 1, NAR's were consistently decreased by infection. Surprisingly, however, the decrease was not statistically significant although it was as large as, for example, that found by Morton and Watson. That the effect of infection was not greater may have been due to some factors ameliorating adverse influences on NAR. For example, the fewer and smaller leaves of diseased plants probably shaded one another less than those of healthy plants, and, as they spread over a smaller area, might have been shaded less by neighbouring plants. Wilting was probably most severe around mid-day when the rate of photosynthesis might have dropped even in healthy plants—the so-called 'mid-day depression'—because of stomatal closure or perhaps temporary accumulation of assimilates (Rabinowitch, 1945). It must be concluded, however, that the decline in dry-matter production following infection is due principally to the decrease in leaf area.

The precise effect on the plant depends on the time of infection and observation. Delaying infection allows fibrous roots and tops to grow considerably before material is diverted to the gall, which sometimes results in larger tops at the time of harvest even though the club caused by late inoculation is finally larger than that caused by early inoculation. Blunck's (1928) recommendation that, to minimize the losses from clubroot, transplants as large as practicable should be used, seems to be a practical application of this result.

No single measurement appears to provide sufficient information to assess severity of disease. The percentage, in clubs, of the total dry weight can be regarded as a measure of the success of *P. brassicae* in appropriating the available nutrients. As clubs grow at the expense of fibrous roots and tops, this figure also gives some indication of the extent to which the plant is crippled. How much its capacity for further growth is impaired is more directly illustrated by the amount of fibrous root per unit of shoot or of leaf, as these are the organs immediately concerned in supporting growth. These figures are obtained only laboriously and are clearly not practicable for every-day assessment of disease severity. Probably the most useful measurements are those made in our experiments on griseofulvin, namely, the fresh weight of tops or of any other organ that would count as yield of agricultural value, and the fresh weight of clubs as a measure of the growth of *P. brassicae*. In assessing by eye how severely a plant is affected by clubroot, the extent of the fibrous root system is probably the most important factor to consider.

ACKNOWLEDGEMENTS

We wish to thank all those who helped in the collection of the results and in other ways, particularly Miss Rosemary Hamley and Miss Jane Jackman (Mrs. Nour), our assistants, and Mrs. M. Macfarlane.

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Structure, Life-history, and Sex Determination of *Prasiola stipitata Suhr*

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With four Plates and six Figures in the Text

ABSTRACT

By means of cultures and cytological investigations *P. stipitata* was shown to have a dimorphic diplo-haploidic life-history, as follows: The diploid spore-forming thalli produce diploid spores ($2n = 12(14)$). The sexual plants comprise a diploid meiosporophyte and numerous haploid gametophytes ($n = 6(7)$). Sex determination is haplogenotypic.

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INTRODUCTION

PRASIOLA is one of the most extensively investigated genera of the Chlorophyceae. No less than 5 monographs are available on the genus (Jessen, 1848; Lagerstedt, 1869; Agardh, 1883; Imhäuser, 1889; Knebel, 1936). In addition, there appeared several papers on its anatomy, development, cytology, &c. (Lagerheim, 1892; Wille, 1906; Sjöstedt, 1922; Hüls-

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bruch, 1939; Yuasa, 1940; Chadefaud, 1951; Copeland, 1955) as well as numerous floristical-ecological reports dealing with this genus.

Considering the great interest shown by phycologists in *Prasiola*, it is surprising indeed that such a conspicuous structure as the gamete-forming thallus could so long remain unobserved. Yet on many herbarium sheets and exsiccata of *Prasiola*, gamete-forming plants clearly show their characteristic pattern (e.g. Desmazières Crypt. France No. 303). Reports by Japanese workers on sexual thalli of *P. japonica* Yatabe (Yabe, 1932; Uda, 1948*a*, *b*; Fujiyama, 1949) were overlooked or ignored by prominent phycologists, who considered them unbelievable. Fujiyama in 1955 published a more detailed description and a photograph of the gamete-forming thallus of *P. japonica*, which is quite similar to that of *P. stipitata*. As to the latter, sections of gamete-forming thalli were figured by Newton (1931) although without a description, with a note to the effect that 'sexual reproduction (is) incompletely known, probably dioecious'. Drew observed the gamete-forming thalli first in 1955. Subsequently, a photograph and description were published by Drew and Friedmann in 1957.

The discovery of sexual plants led to attempts to establish the life-history of *Prasiola*. Fujiyama (1955), working with *P. japonica*, concluded that *Prasiola* is a haplont, the reduction division taking place at the germination of the zygote. His results are not in agreement with our own.

The present investigation was suggested by the late Dr. K. M. Drew-Baker. Till her untimely death in the summer of 1957, she followed the work with great interest and offered valuable criticism. The author is deeply and sincerely grateful for the time he was able to spend working together with Dr. Drew-Baker and to benefit from her extraordinary scientific insight and personality.

MATERIAL AND METHODS

Material was collected periodically from November 1956 to January 1958, at different points of the North Welsh and Manx coasts. In North Wales thirteen collections were made in Beaumaris and Bangor and one in Rhosneigr. The Isle of Man was visited twice, in early April and again in the middle of May 1957, and collections were made at Port Erin, Port St. Mary, Calf Sound, Perwick Bay, and Fleshwick Bay. Each time thalli were collected at different levels of the intertidal belt, thus comprising the whole vertical range of *P. stipitata*. In all, 128 samples were taken.

The plants were partly fixed on the spot, partly transferred living to the laboratory in thermos flasks pre-cooled with ice which was removed immediately before collecting. The algae were fixed in formalin-alcohol (Drew, 1934), formalin-acetic-alcohol, acetic-alcohol, Fleming-Benda's and Zenker's fluids. Best results were obtained with formalin-alcohol and formalin-acetic-alcohol. Fleming-Benda's fluid showed no advantage over these in fixing cytological details, a cumbersome bleaching was necessary and stainability was reduced. Zenker's fixative was entirely unsatisfactory and it caused shrinking of the protoplasts.

Microtome sections were stained with Heidenhain's hematoxylin. For whole mounts, the following modified aceto-carmine method gave best results: The thalli, fixed in formalin-alcohol, formalin-acetic-alcohol or acetic-alcohol, were transferred through alcohol-stages in distilled water, and subsequently mordanted for several hours in freshly prepared, *c.* 4 per cent. iron alum solution. After rinsing, they were heated in 4 per cent. carmin and left in the stain for 5–7 days. The purple-black thalli were then destained in 45 per cent. acetic acid, by boiling if necessary. After thorough washing in distilled water they were passed through alcohol stages and xylol and mounted in balsam. Whole mounts were also successfully stained in Drew's brazilin (see Appendix).

Cultures were made in Petri-dishes on silica gel plates, prepared by Pringsheim's method (1946). Single thalli were isolated in a drop of culture solution, as described by Drew (1952) although cover slips were used instead of slides. Four culture media were used: (1) Lewin's medium for *Prasiola stipitata* (Lewin, 1955); (2) Drew's medium for marine algae (publ. in Krishnamurthy, 1959); (3) Lewin's medium with vitamins of the B-complex added; (4) Drew's medium with trace elements added (Burkholder and Nickell, 1949). Each day the culture solution was poured off from the plants. After several hours it was replaced by a fresh solution, thus imitating, to a certain extent, the conditions of the intertidal belt. The culture dishes were immersed in a water tank cooled to 5–7° C. and were illuminated for 8 hours a day by a combination of fluorescent tubes and incandescent bulbs.

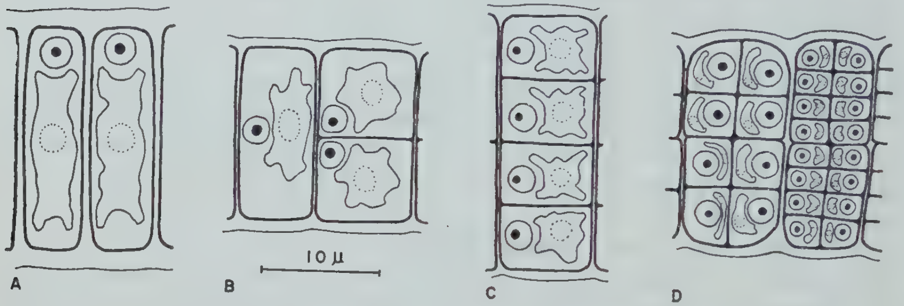
The cultures were not sterile but remained practically unialgal when kept in Lewin's medium. In Drew's solution, as well as in Lewin's medium with added vitamins there were many cases of infection by diatoms. The lack of vitamins presumably inhibited the growth of diatoms (in Drew's medium, vitamins are probably present from the soil extract). As far as the growth of *Prasiola* is concerned, the addition of vitamins caused no noticeable difference.

GENERAL FEATURES OF THE THALLUS

The young vegetative thalli of *P. stipitata* develop either into spore-forming or into gamete-forming plants. The formation of the two kinds of reproductive cells takes place in the apical region of the thallus and the basal portion of adult generative (spore-forming or gamete-forming) plants retains its vegetative character, morphologically resembling young thalli.

The vegetative regions of thalli are one-layered. In the generative thalli, both spore-forming and gamete-forming, periclinal divisions occur (Text-figs. 1, 2). Viewed from the surface of the thallus, the cells appear arranged in a characteristic pattern of areoles, each of which corresponds to a mother cell of previous divisions. Different regions of the thallus show characteristic variations of this pattern (Plate 1).

The thallus of *Prasiola* is characterized by a double wall, composed of two distinct layers of different chemical composition. Each cell has an *inner, individual membrane*, embedded in the outer layer which forms a continuous sheath. This covers the whole thallus and penetrates between the inner membranes, separating the cells by anticlinal septa (Text-fig. 1). The inner wall consists of cellulose, impregnated with pectinous substances. When acted upon with iodine-potassium iodide, after treatment with 70 per cent. sulphuric acid, the inner cell wall turns blue. It stains in Ruthenium red and dissolves readily in cupric-oxide ammonia. The chemical nature of the outer cell wall could not be established with the usual microchemical methods. A similar membrane structure was recorded in *Prasiola crispa* (Knebel, 1936) and *P. fluviatilis* (Hülsbruch, 1939; Rejment-Grochowska, 1952). The occurrence of a double cell wall is known in several green algae. Brand, who described it in *Cladophora* in 1901, called the outer membrane-layer *coating lamella* (decklamelle), a term which seems more appropriate than 'cuticle', somewhat confusing in algae. In *P. stipitata* the coating lamella has a special role in reproduction, for when the stage of gamete liberation is reached the thin anticlinal septa decompose first, while the outer coating lamella persist as a bladder-like structure into which the gametes are discharged. It is there, too, that fusion takes place (Plate 2, Fig. 5).



TEXT-FIGURE 1. *Prasiola stipitata* Suhr, cells in cross-sections of adult thalli. Individual cell membranes are indicated by black line, the coating lamella and the anticlinal septa are kept white. A: Diploid vegetative cells. Polarity is apparent from the nuclei lying in one plane and the chromatophores in another (cf. diagrammatic representation in Text-fig. 2, ① and ②). B and C: Spore formation (diploid) by anticlinal divisions. Viewed from the surface of the thallus the nuclei lie beside the chromatophores (cf. diagram in Text-fig. 2, ③). D: Haploid gametophytic cells, to the left a female, to the right a male sarcinoid group. From the position of nuclei and chromatophores it is evident that the last division has been anticlinal. (All three orientations of the cells shown in Text-fig. 2 occur in haploid tissues.) Microtome sections stained with Heidenhain's hematoxylin.

The chromatophore of *Prasiola* has been investigated on several occasions. Its central position has repeatedly been stressed and far-reaching cytological, systematical, and phylogenetical conclusions were based on this characteristic (Fritsch, 1935; Chadefaud, 1936, 1951, 1954, 1957; Gams, 1958; Meyer, 1951, 1952; Vischer, 1953). In *P. stipitata*, however, the chromatophore shows considerable variation in shape. Even its central position is not absolutely fixed. In different cell types, the chromatophore assumes three typical forms: (1) In spores as well as in young germlings developing from spores or from zygotes and finally in the basal cells of adult thalli the chromatophore corresponds to the 'axile type' chromatophore, as defined by Starr (1955): Its central mass bears 'variable processes which extend peripherially in an irregular manner. The processes extend in all directions from the central mass, with the exception of a small area on one side in which the nucleus is located'. Starr's definition is based on the Chlorococcal genus *Trebouxia*. As a matter of fact, the spores of *Prasiola* are strikingly similar to vegetative cells of *Trebouxia* (Text-figs. 3 A, B; Plate 1, Figs. 1, 7). (2) In diploid vegetative and haploid cells the processes of the chromatophore resemble lobes. They are much shorter in relation to the central mass than in the previous type. Accordingly, the nucleus does not lie between the processes, but flanks the whole of the chromatophore (Text-figs. 1 A, 3 E; Plate 1, Figs. 2–5). (3) In gametes the chromatophore is parietal, flattened, and irregular in shape (Friedmann, 1959). In gametophytic tissues transitional stages occur between types 2 and 3 (Text-figs. 3 C, D; Plate 1, Fig. 8, Plate 2, Figs. 1, 2). The chromatophore always bears a single pyrenoid.

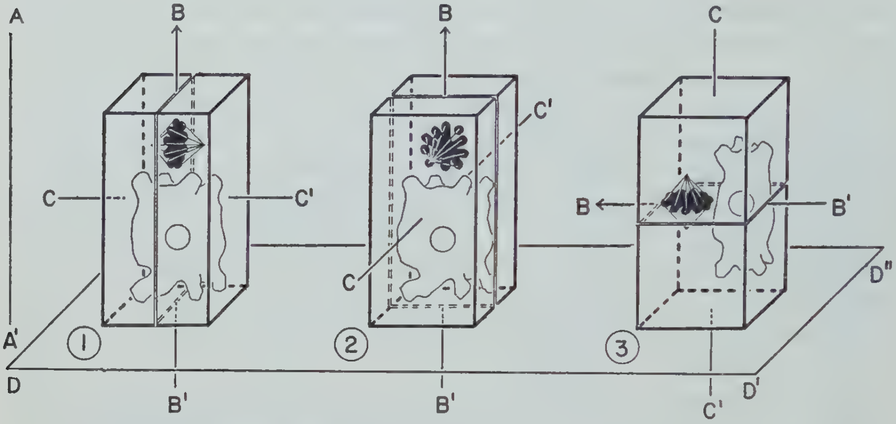
THE VEGETATIVE THALLUS

Vegetative thalli are diploid. They develop from spores or zygotes. Germination is similar in both cases and agrees well with Imhäuser's (1889) description of spore germination.

The cells of the basal portion of the thallus are relatively large and nearly isodiametric (Plate 1, Fig. 1). With this exception, vegetative cells have the shape of elongated prisms (Text-fig. 1 A), their (geometrical) *long axis* being perpendicular to the plane of the thallus. In these cells the (morphological) *cell axis*, defined as the line which passes through the nucleus and the chromatophore, coincides with the long axis. The *division axis* is defined as the line joining the centre of the daughter cells produced by cell division, and is perpendicular to the cell axis. In subsequent divisions, the division axis turns through a right angle in the two dimensions of the thallus plane (diagram in Text-fig. 2). These changes in orientation lead to the formation of the strikingly regular, rectangular cell pattern (Text-fig. 1 A; Plate 1, Figs. 2–5).

Diploid vegetative cells are polarized and in the thallus all cell axes show a uniform orientation. Thus, in any given thallus, all the nuclei lie in one plane, all the chromatophores in another (Plate 1, Figs. 3, 4). However, this polarity is restricted to the elongated cells of the diploid vegetative thallus. It is not present in the basal cells and in the multi-layered spore-forming or haploid tissues.

Cell polarity reveals itself already in young germlings. In these uniseriate filaments the nuclei are orientated strictly in a line running along the filament (Text-fig. 3 B). As a result of cell polarity, the thalli of *Prasiola* are dorsiventrally symmetrical, and not—as would appear from macroscopic examination—bilateral.



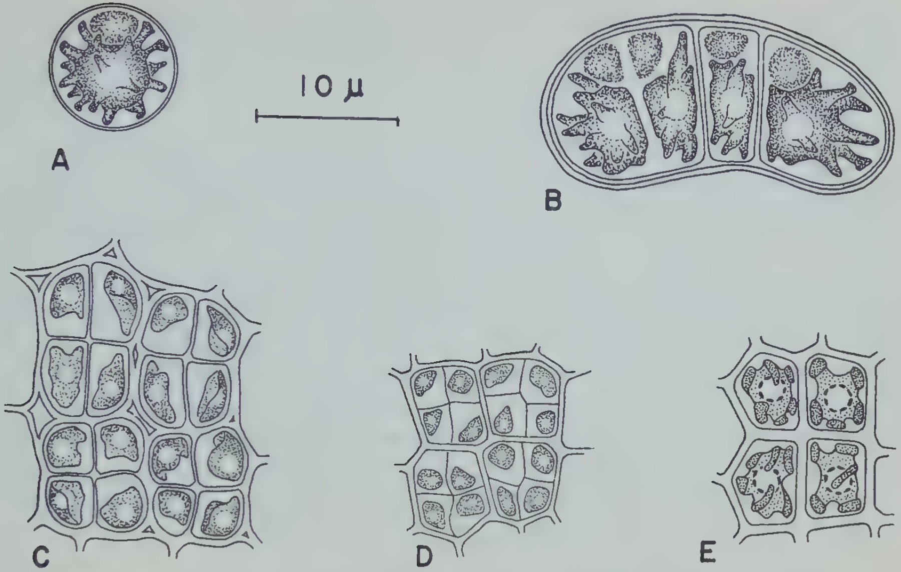
Text-figure 2. *Prasiola stipitata* Suhr, diagram of possible positions of axes and planes in the cells of adult thalli. A A′: Direction of the long axes. B B′: The morphological cell axis which passes through the nucleus and the chromatophore. C C′: The division axis which joins the centres of the daughter cells produced by cell division. This is parallel with the spindle axis, and perpendicular to the plane of division. D D′ D″: Plane of the thallus. || (Double line): Plane of cell division. ① and ②: Diploid vegetative and haploid cells as in Text-fig. 1 A and D. ③: Diploid spore-formation and haploid cells as in Text-fig. 1 B and C. For further explanation, see text, p. 575.

The Spore-forming Thallus

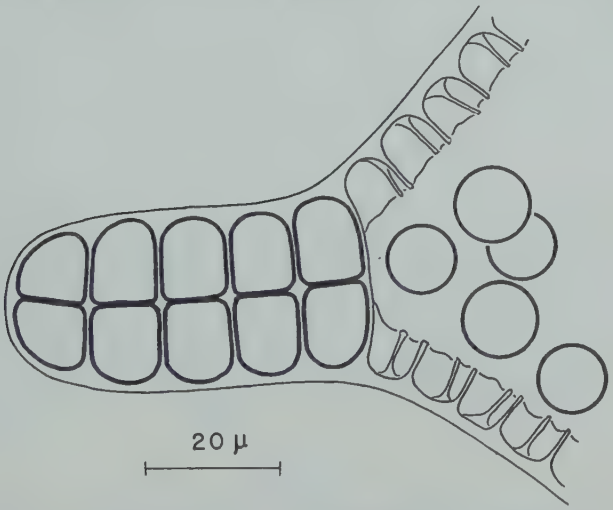
Because of the rather complex homology of sporophyte and gametophyte in *Prasiola*, the non-committing and self-explanatory terms *spore-forming thallus* and *gamete-forming* (*sexual*) *thallus* resp. *plant* are used to designate whole thalli.

Spore-forming thalli are diploid. Generative thalli remain vegetative in their lower portion and the formation of reproductive cells takes place in their upper part. In spore-forming plants a *spore-forming zone* and, over this, a *spore mass zone* can be distinguished in the upper part of the thallus. The cells in the spore-forming zone undergo internal structural changes as the cell axis turns through a right angle. Thus, it becomes perpendicular to the long axis and parallel to the thallus plane. Chromatophore and nucleus lie side by side in the centre of the cell on the same level. Consequently, the division-axis becomes perpendicular to the thallus plane and thus coincides with the long axis. As a result of this, divisions become periclinal and a two- to four-layered tissue is formed (Text-figs. 1 B, C, 2; Plate 1, Fig. 6).

The daughter cells of periclinal divisions are nearly isodiametric and their chromatophores assume the shape characteristic of spores. In case of a low rate of cell division, periclinal divisions may not take place, and the whole spore-forming thallus may then remain unilayered. Even so, the change in



TEXT-FIGURE 3. *Prasiola stipitata* Suhr, living cells. A: Spore, chromatophore axile. B: Young germling, chromatophores axile. Polarity is apparent from the nuclei being arranged in a line running along the filament. C, D: Female and male haploid cells. The chromatophores are nearly flattened and in a parietal position. E: Diploid vegetative cells, chromatophores lobate. In figs. C, D, and E the nuclei are not shown.



TEXT-FIGURE 4. *Prasiola stipitata* Suhr, spore discharge. Hand section through a living spore-forming thallus of the variegated type. By the swelling of the spores the anticlinal septa of the coating lamella are torn. The cell contents are not drawn.

the orientation of the cell axis is apparent from the relative positions of chromatophore and nucleus.

Periclinal divisions are followed by the maturing of spores as cells round off and increase in size. Consequently, the thin anticlinal septa of the coating lamella are torn and mature spores lie in the persisting bladder-like structure formed by the lamella. By the gradual decomposition of this 'bladder' the spores are liberated (Text-figs. 3 A, 4; Plate 1, Fig. 7). The spore-forming process thus proceeds basipetally.

Morphologically, two distinct types of spore-forming thalli can be distinguished. When spore liberation keeps up with the activity of the spore-forming zone the spore-mass zone occupies but a narrow marginal strip at the apex of the thallus (*marginal type*) (Plate 3, Figs. 1, 2). On the other hand, when sporogenous activity sets in simultaneously at many points, the spore-mass zone spreads over a considerable part of the thallus. Maturing spores tear the anticlinal walls of the lamella in many points. Scattered over the thallus, these form small pocket-like dilatations, filled with loose spores. In this case the spore-mass zone has a characteristic variegated appearance (*variegated type*) (Plate 3, Figs. 3, 4). Transitional forms between marginal and variegated spore-forming thalli may occur but are not frequent. By means of cultures it could be demonstrated that the type of spore formation is determined by external factors (p. 580).

THE GAMETE-FORMING (SEXUAL) THALLUS

The lower, vegetative portion of the gamete-forming thalli is diploid. Morphologically, it is indistinguishable from the lower portion of a spore-forming plant. However, the upper cells of the vegetative portion undergo reduction division (meiotic zone) and the daughter cells subsequently develop into a haploid tissue. Both first and second meiotic division take place in the plane of the thallus. The subsequent haploid mitoses are marked by the division axis regularly turning through a right angle in the three main directions (Text-fig. 2). As a result, a multi-layered haploid tissue is produced. Within the haploid tissue the cells are arranged in very regular sarcinoid groups. These groups are separated from each other by anticlinal septa of the coating lamella (Text-fig. 1 D). As the haploid cells in the sarcinoid groups undergo a number of divisions, the size of cells in the individual groups diminishes towards the apex of the thallus. The haploid tissue appears divided into lighter and darker areas with rectangular boundaries, arranged in an irregular 'patchwork' pattern (Plate 2, Figs. 3–8; Drew and Friedmann, 1957). These light and dark areas contain male and female cells respectively. The difference in shade is due to difference in the size of chromatophores, the female cells having the larger ones (Text-figs. 3 C, D; Plate 1, Fig. 8, Plate 2, Fig. 1). The female cells themselves are larger, too, and accordingly the number of cell layers in female areas is smaller than that in male ones. While the male areas comprise up to 16 cell layers, the female areas have half this number—not more than 8 layers.

Close examination of the 'patchwork' pattern reveals a regular system of paired male and female areas. Each pair comprises adjacent unisexual areas of nearly equal size in the shape of a rectangular parallelogram (Plate 2, Figs. 7, 8). The size of different pairs, however, varies greatly even in the same plant (Plate 2, Fig. 6).

A study of the development of this peculiar arrangement shows that each pair of areas originates from the first meiotic division of a mother cell. Transitional stages between meiotic cells and differentiated pairs of unisexual areas can be observed in young gamete-forming plants which possess an active meiotic zone. Apparently, the second meiotic division does not differ essentially from the following haploid mitoses and thus each meiosis gives rise to a single pair of areas.

At maturity each haploid cell develops a single gamete. By the dissolution of the inner cell wall, the gametes are liberated into the space formed by the persisting bladder-like lamella, and it is there where their fusion takes place (Plate 2, Fig. 5). As a rule, numerous gametes, several hundreds or thousands, are discharged at once. In a single gamete-forming plant all stages of gamete development may be observed simultaneously from the meiotic zone to the mature gametangia at the apex.

SPORE FORMATION IN SEXUAL THALLI

In gamete-forming thalli the exceptional formation of spores may occasionally be observed (Plate 2, Fig. 2). The spores occur singly or in small groups, between normal-looking haploid cells. The occurrence of spores in sexual plants is more frequent towards the end of the growing season of *P. stipitata*, when liberation of gametes ceases. Indeed, there appears to be a general tendency, towards the end of the season, to form resting cells, as in decaying thalli, several of the diploid vegetative cells, too, assume spore-like characteristics recognizable in chromatophore shape and persist between dead cells (Plate 3, Fig. 5). By means of cultures and cytological staining it could be shown that these spores germinate into diploid plants.

In sexual plants which were collected at their natural habitat, spore formation can be induced by keeping them dry in cultures. Also under natural conditions it is probably dryness which is decisive for the occurrence of spore formation in sexual plants. At the end of the growing season of *P. stipitata*, when this type of spore formation is relatively frequent, the seasonal lowering of the highest high-tide level takes place, exposing the *Prasiola*-belt to prolonged desiccation.

CULTURES

Cultures from spores. The spores which were collected at different times and different localities readily germinated in culture. They did not pass a resting stage after their liberation. In each case germination was practically complete.

A series of cultures was started from the spores liberated by a spore-forming thallus of the marginal type, collected at Bangor on November 14, 1956. On January 29, 1957, 40 germlings were transferred to different culture media. By April or May 1957 all of these had developed into spore-forming plants.

There was a clear correlation between the type of spore formation and the culture medium employed. In Drew's medium, with or without added trace elements, there developed the variegated type of spore formation. In Lewin's solution the marginal type appeared. Only two exceptions were seen, one thallus in each medium. These results are in accordance with those of Lewin (1955) who reports spore formation, evidently of the marginal type, in his culture medium.

The spores obtained in culture from both types of thallus germinated normally. In August 1957 the cultures were discontinued and the sporelings, still in a vegetative condition, were fixed. Cytological investigation revealed them to be diploid.

The results of cultures are summarized in the following table:

Germination in: L Si
Mother plant: MARG
40 germlings

Germlings transferred to:	D Si	L Si	DT Si	D Co	D Co	L Co	L Co
Number and type of fructificating thalli:	13 VAR	13 MARG 1 VAR	9 VAR	1 VAR	1 MARG	1 MARG	1 MARG

L = Lewin's medium
D = Drew's medium
DT = Drew's medium + trace elements
Si = Silica gel plate
Co = Cover slip with drop of culture fluid on
MARG = Marginal type of spore formation
VAR = Variegated type of spore formation

Culture of spores from sexual plants. Spores which appeared in the haploid tissue of gamete-forming plants were germinated in two silica-gel cultures in Lewin's medium. The mother thalli were collected in Beaumaris at the end of the growing season (May 5, 1957) at a time when gametes were no longer liberated. The spore germlings developed into normal vegetative thalli. Cytological staining showed them to be diploid (Plate 4, Fig. 2).

Culture of zygotes. Zygotes were obtained in culture by isolating mature sexual thalli collected in Beaumaris on November 14, 1956. After liberation of the gametes the zygotes, obtained thus by autogamy, were transferred to silica-gel plates and cover-slip cultures. The media of Drew and Lewin were employed. The germination of the zygotes as well as their subsequent development were very much slower than in spores germinated under similar conditions. The zygotes developed into vegetative thalli which, throughout the 9 months during which the cultures were observed did not produce any fructification. The thalli were of a pale colour and of a curled, somewhat abnormal shape. They were similar to the thalli which Lewin obtained in cultures of spores, under suboptimal nutritional conditions (Lewin, 1955, Fig. 8). Subsequent cytological investigation showed abnormal multinucleate cells. The nuclei of normal cells were diploid.

The filamentous stage in culture. In two cultures of spores the filamentous '*Hormidium*' stage, generally attributed to *Prasiola*, appeared between germlings normally developing into sporophytic thalli. In all, three filaments were found in two culture plates. The mother plants were collected in Beaumaris on November 14, 1956. Germination was on silica plates in Lewin's medium. The filaments were transferred to new plates in Lewin's and Drew's medium. They showed the characteristic features of the '*Hormidium*' state and also developed rhizoids, but no fructification was detected during 9 months of culturing. Artificially detached segments could be transferred into new cultures where they developed readily. As a possibility of infection could not be excluded, the appearance of filaments in cultures of *P. stipitata* cannot be regarded as an absolute proof of their being part of the life-history of this alga.

SEASONALITY OF DEVELOPMENT AND ZONATION OF SPORE- AND GAMETE-FORMING PLANTS

A full report of the ecological and phenological data on *P. stipitata* will be published elsewhere. However, as some of these observations are of importance in the life-history of this alga, they are briefly reported below.

The development of *P. stipitata* is seasonal. In Beaumaris young germlings appear in October and the thalli die the following July. In May gamete liberation ceases and towards the end of the growing season spore formation is the only form of reproduction.

Within the *Prasiola*-belt there is a conspicuous zonation of spore-forming and sexual plants. The former grow at the highest and the latter at the lowest level of the belt, with a continuous gradient in between. This situation was found to occur in each locality. As *P. stipitata* grows in the intertidal belt, the vertical zonation of spore-forming and sexual plants is apparently due to differences in exposure to the tidal waves.

CARYOLOGY

The caryological observations reported below were carried out in order to complete the life-history of *P. stipitata.* They do not claim to be an exhaustive account of the caryology of this alga.

The resting nucleus varies considerably in size. It is particularly large in spores. There does not appear to be a correlation between the size of the nucleus and the haploid or diploid status of the cells, although the nuclei are generally smaller in haploid cells, due to the smaller cell-size in haploid tissues. The stained resting nucleus contains only a few small stained granules. Also, there is a clear nuclear membrane and a single nucleolus (Plate 4, Fig. 10).

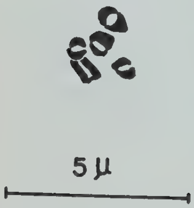
Mitosis in diploid cells. Diploid mitoses were observed in spore germlings, in young vegetative thalli, in vegetative cells of both spore-forming and gamete-forming thalli, as well as in germlings of spores exceptionally produced by sexual plants.

The late prophase is favourable for chromosome counts, as in this stage the very small chromosomes show distinctly. Even in good late prophase stages, chromosomes could not be counted exactly, and a margin of 12 (14) had to be allowed. At this stage the smaller chromosomes are about 0·25–0·3μ in diameter, the larger ones about 0·35–0·4 $\mu \times$ 1–1·2 μ.

At metaphase, the chromosomes are crowded on the plate and their individuality and number is nearly indistinguishable. A spindle is apparent. In anaphase and telophase the chromosomes form a highly condensed mass and as a rule the spindle is bent. As diploid vegetative thalli are unilayered, diploid mitotic spindles always lie in the plane of the thallus (Text-fig. 2).

Periclinal divisions which lead to spore formation are essentially also diploid mitoses. Nevertheless, these divisions differ from the pattern described above. Periclinal divisions are preceded by a reorientation of both cell- and division axes (p. 576). Thus, unlike the vegetative divisions, the spore-forming division proceeds while the spindle lies in the direction of the long axis of the cell. Viewed from the surface of the thallus, the nucleus lies *beside* the chromatophore. Diploid cells in spore formation differ from vegetative ones also in the morphology of their chromosomes. The chromosomes are generally larger than in vegetative mitoses, at metaphase and anaphase they are conspicuously longer and are arranged in a loose tangled mass (Text-fig. 1 B, C; Plate 4, Fig. 9).

Meiosis. Reduction divisions are numerous in young gametophytic thalli on the borderline between diploid vegetative and haploid tissues (meiotic zone). In meiotic prophase, the nucleus considerably increases in size. At first a fine network of chromonemata and a distinctive nucleolus are present (Plate 4, Fig. 11, 12). Subsequently, the chromonemata thicken and their coiling may occasionally be observed: pachytene (Plate 4, Fig. 13). At this stage the nucleolus appears less distinctive. In diplotene (Plate 4, Fig. 14) the bivalents are clearly discernible. At diakinesis (Text-fig. 5; Plate 4, Fig. 15) the nuclear membrane and nucleolus disappear and the pairs of chromosomes are highly contracted. At metaphase I, the individual elongated bivalents appear distinguishable. They are orientated already on the spindle. Their elongated shape is apparently due to strain and immediately after their separation (early anaphase) the chromosomes contract and assume a nearly isodiametric shape (Plate 4, Figs. 17–19). As a rule, a single pair of chromosomes show a precocious separation which appears already in the metaphase and their precocious movement is still conspicuous at anaphase I. In late anaphase I the chromosomes appear elongate and more loosely arranged than at mitotic anaphase. The second meiotic division is similar in appearance to mitosis.



TEXT-FIGURE 5. *Prasiola stipitata* Suhr, end of diakinesis. The drawing shows the same cell as in Plate 4, Fig. 15.

Both meiotic divisions take place in the plane of the thallus, i.e. in a similar orientation to vegetative diploid mitoses (Plate 4, Fig. 4). Periclinal divisions set in only after meiosis in the haploid tissue.

Mitoses in haploid cells. Mitoses in haploid cells are similar to those in diploid ones. Cells in late prophase show 6 (7?) very small chromosomes. On the metaphase plate, chromosomes usually lie closely together and are often unfit for counts. The spindle in telophase is usually bent (Plate 4, Figs. 5–8). In haploid tissues divisions are cut in all three possible planes (Text-fig. 2).

Comparison of mitotic division figures in diploid and haploid cells reveals a conspicuous difference in the size of the chromosome mass. The volume of chromatin seems to be quite constant and can be roughly estimated in haploid nuclei as being half of that in diploid ones. The anaphase and telophase are favourable for such observations (Plate 4, Figs. 1, 5, 6). Thus, it is possible to judge, with a fair certainty, about haploidy or diploidy of particular cells from the size of the chromatin mass. Considering the great difficulties in chromosome counts in *Prasiola*, this method might often prove useful. It is worth while to compare these images, e.g. with Dermen's (1951) photographs of mitotic anaphases in diploid and tetraploid cells in apple, which show a similar ratio in the chromatin volume.

In meiotic cells the chromatine mass increases conspicuously. The reduction of chromatin volume is apparent between the first and second divisions (Plate 4, Fig. 4).

No conclusions can be drawn from these rather tentative observations as to the quantity of chromatin in the nuclei. Nevertheless, a comparison with the results of exact measurements, as discussed by Swift (1953) might be of interest.

Diploid cells in haploid tissues. In some cases a chromosome number higher than haploid was counted in individual cells of typical haploid tissues. The exact chromosome number could not be established with certainty, but at least 10–12 were counted (Plate 4, Fig. 3). In the same thallus, other cells showed the normal haploid chromosome number. These apparently diploid cells might give rise to the diploid spores which occur occasionally in gamete-forming plants. No indication could be found concerning the mode of origin of such exceptional diploid cells within haploid tissue.

Pyrenoid division. By cell divisions, the rather conspicuous division of the pyrenoid can be observed (Plate 4, Fig. 16). It can precede or lag behind the division of the nucleus. There is no need to describe it in detail, as the process as observed in *P. stipitata* is essentially the same as that described by Yuasa (1940) for *P. japonica.*

DISCUSSION

(*a*) *The spore-forming thallus*

The spore-forming thallus of *P. stipitata* is a diploid sporophyte producing diploid spores. To emphasize their diploidy, the terms *diplosporophyte* and *diplospore* should be applied to them.

The terminology in use in literature dealing with the reproductive cells in the genus *Prasiola* is rather confused. Therefore the use of the term *spore* for non-sexual reproductive cells in this genus requires some explanation. In *P. stipitata* there is no clearly defined sporangium liberating spores by dehiscence of its membrane. Instead, the spores are covered by the coating lamella which is common to the whole thallus. Nevertheless, contrary to the views of some recent authors, the non-sexual reproductive cells cannot be regarded as akinetes. Akinetes are essentially a form of vegetative reproduction. One cannot but agree with Fritisch (1935, pp. 38–39) who holds that while in vegetative reproduction 'portions of the plant body become separated off to give rise to new individuals without any obvious changes in the protoplasts', in asexual reproduction 'we have, on the other hand, a rejuvenation of the protoplasts of certain cells, the latter being commonly associated with division of the protoplast'. The 'rejuvenation of the protoplasts' in *P. stipitata* clearly manifests itself in the structural changes which take place in the cells prior to spore formation, i.e. in the translocation of cell- and division-axis, periclinal divisions and particular caryological behaviour during divisions. Beside these, the absence of a characteristic sporangium seems to be only of secondary importance, as the peculiar structure of the *Prasiola* thallus, with its coating lamella, makes a clear morphological distinction of the membranes of individual cells difficult. The whole structure rather suggests a reduction of 'individual' sporangia, the types of *P. furfuracea* and *P. crispa*, of *P. mexicana*, and finally of *P. stipitata* representing three successive stages in this reduction. Thus, in *Prasiola furfuracea* (Mert.) Menegh. and in *P. crispa* (Lightf.) Menegh., true sporangia with dehiscent membranes are present. They develop by germination of akinetes, as described by Wille (1906) and Knebel (1936). (In *P. furfuracea* the presence of sporangia could be confirmed on fixed material obtained from Lake Erken, Sweden.) *P. mexicana* Liebm. apud J. Ag., does not form sporangia with dehiscent walls but spores are arranged in conspicuously regular groups of eight, each group deriving from a single mother cell. These groups are apparently homologous with sporangia and Lagerheim (1892) rightly designated them as such. Fritsch (1935, p. 220) calls them 'tetraspores' presumably owing to the misinterpretation of Lagerheim's figures which clearly depict groups of *eight*. (The correctness of Lagerheim's figures could be confirmed on fixed material of this species, obtained from Butte Creek, California.) In *P. stipitata*, the two or four spores in a transversal row might be regarded as homologous with a sporangium. They derive from a single mother cell and are surrounded as a unit by anticlinal septa of the coating lamella (Text-fig. 1 B, C). It does not alter the situation essentially when, in case of low division frequency, no periclinal divisions occur and each sporangium mother cell develops into a single spore. It should, further, be pointed out that the non-sexual reproductive cells of *Prasiola* do not possess a thickened membrane and do not require a resting period before germination as Chlorophycean hypnospores do. This was demonstrated in *P. stipitata* in culture experiments (p. 579, further Lewin, 1955) and was shown in *P. japonica* by Fujiyama (1955) and in *P. mexicana* by Lagerheim (1892).

It can be concluded, therefore, that the non-sexual reproductive cells of *P. stipitata*, as well as of *P. mexicana* and probably of *P. japonica* can be regarded as spores. On the other hand, akinetes do occur in *P. furfuracea* and *P. crispa* and they germinate as sporangia.

(*b*) *The gamete-forming* (*sexual*) *thallus*

The rather unusual structure of the sexual thallus may be interpreted in one of two ways. (1) The haploid tissue, or the single (unisexual) areas, or parts of them, are plurilocular gametangia of the diploid gametophyte and thus, *P. stipitata* should be a diplont. (2) The haploid section of the thallus represents a separate nuclear and somatic phase, i.e. the gametophytic.

There is one important argument against the first interpretation and that is the occurrence of haploid mitoses between meiosis and gamete formation. It can be argued, however, that in algal diplonts in the orders Siphonales (*Bryopsis*, Zinnecker, 1935, *Codium*, Schussnigg, 1950) and Siphonocladales (*Valonia*, Schechner-Fries, 1934), further in the Fucales (e.g. *Fucus*, Strasburger, 1897, &c.) haploid mitoses generally follow meiosis in gametangia.

Even so, there are more points in favour of the second interpretation. The haploid mitoses in the diplonts mentioned above can also be regarded as a reduced haploid gametophytic phase (for possible interpretations in Fucales see Kylin, 1940). But even if this view is not accepted and it is assumed that haploid mitoses appeared secondarily in the life-history of these algae, this can hardly be applied to *Prasiola*. For in all the cases mentioned, the haploid mitoses occur in morphologically clearly defined gametangia and are but a few in number. In *Prasiola*, on the other hand, very numerous haploid mitoses set in. They do not occur in morphologically clearly defined gametangia, but a well-developed haploid tissue is formed which even shows a higher anatomical organization than the diploid. These conditions certainly satisfy the criterion of a nuclear phase, defined by Drew (1955) as a 'state of an organism characterized by mitotic divisions all showing the same chromosome number'.

It seems, therefore, justified to interprete the haploid tissue of the sexual thallus as the gametophytic phase of *Prasiola*. Accordingly, sexual thalli should be regarded as complexes, comprising a diploid and haploid phase. Morphologically, cytologically, and genetically the diploid phase, i.e. the basal and vegetative zones of the thallus, is actually identical with the corresponding zones of the diplosporophyte. It was also shown that, given suitable conditions, it has the potentiality to form diplospores. Thus the diploid phase of the sexual plant should be interpreted as a sporophyte forming haploid spores by reduction division. By contrast to the diplosporophyte and diplospores, the terms *meiosporophyte* and *meiospores* can be appropriately used for them. The meiospores are not discharged. Instead, they develop directly into gametophytes (haploid phase, gamete-forming zone of the sexual thallus). Each unisexual area in a pair (Plate 2, Figs. 7, 8) is regarded then as a single gametophyte and the gamete-forming zone itself represents the total progeny of the meiosporophyte. The gametophytes remain attached to the mother meiosporophyte and are covered by its coating lamella. It should be noted here that diplospores too often show a tendency to germinate while still attached to the mother diplosporophyte (Plate 3, Fig. 1, further Jessen, 1848, Tab. II, Fig. 11).

The morphological definition of single gametangia in *P. stipitata* is even more uncertain than that of sporangia. Sarcinoid cell groups (Text-fig. 1 D) can be regarded as plurilocular gametangia, or perhaps more appropriately, each mature haploid cell acts as a gametangium discharging a single gamete.

A further point should also be considered in this connexion. Winkler (1942) and Poljansky (1958) pointed out that in evaluating life-histories, the individuality, i.e. the physiological independence of the different phases, has to be taken into account. Accordingly, the single gametophytes of *P. stipitata*, though independent individuals from genetical and developmental points of view, are physiologically dependent on the meiosporophyte. Looked at from a physiological point of view, the sexual thallus as a whole represents a single individual, equivalent to a diplosporophytic thallus. These conditions are expressed in the proposed symbolical designation of life-history (p. 589).

(*c*) *The filamentous stage of* Prasiola

The filamentous '*Hormidium*-stages', often assumed, or suspected, to be a part of the life-history of *Prasiola*, are comprised in the form-genus *Rosenvingiella* Silva (1957) (= *Gayella* Rosenvinge). As recently pointed out by Waern (1952) the reasons given by Knebel (1935) for separating *Prasiola* and *Rosenvingiella* are not quite conclusive. As for *P. crispa* (Lightf.) Menegh. and *P. velutina* (Lyngb.) Wille, their connexion with filamentous stages was clearly demonstrated by Knebel and Wille. Waern's observations on natural habitats of *P. stipitata* make it appear probable that a filamentous stage belongs to this species too. The results of present investigations seem to confirm this, although, as already mentioned, proofs were not absolutely conclusive because there was a possibility that the cultures were infected by alien spores.

(*d*) *Sex determination and formation of sexual thalli*

The results of the present investigations suggest that sex is determined in *P. stipitata* haplogenotypically. Proofs of haplogenotypic sex determination can be furnished either by genetical methods, namely by tetrad-analysis as first applied by Schreiber (1925) or by direct cytological demonstration of sex-chromosomes. As to the first, in *P. stipitata* there is no need to culture the isolated progenies of meiotic tetrades as in this organism the progenies of meioses can be traced topographically in the thallus. The pairs of male and female areas, each of them originating from a single meiocyte, clearly show the 1:1 sex ratio thus supplying genetical evidence for a haplogenotypic sex determination.

As to cytological evidence, the presence of sex-chromosomes have not been definitely established. However, the occurrence of a precociously separating bivalent at metaphase I and anaphase I is certainly suggestive. Further observations might prove that this bivalent is actually the sex chromosome bivalent.

While sex is determined by a genetic mechanism, it is an entirely different question as to which factors induce reduction division and thereby control the formation of sexual plants. Very little is known about these mechanisms. It seems that in *P. stipitata* the onset of reduction divisions is triggered by a combination of external factors such as those brought about by certain tidal conditions. The failure to get sexual plants from germlings in culture might be due to the culture conditions employed being different from those required for inducing meiosis. A detailed discussion of this problem will be given in another paper.

(*e*) *Chromosome number*

In haploid cells, 6 distinct chromosomes were counted. This seems to be the correct number, although the possibility of the presence of 7 chromosomes cannot be excluded. In diploid cells, 12(14) chromosomes were counted. Present findings are in accordance with Sarma's (1958) results, who reports 12–14 chromosomes in *P. stipitata.*

A further problem which is still unsolved concerns the appearance of exceptional spores in haploid tissues. As these spores in culture germinated into thalli whose diploid chromosome number could be established beyond doubt, the spores themselves can safely be considered as being diploid. Moreover, they are morphologically identical with normal diplospores. The diploid (or nearly diploid?) cells with 10–12 chromosomes which sporadically occur in haploid tissues might possibly give rise to spores, although no evidence for this could be found. As to the origin of diploid cells in haploid tissues, they might derive directly from diploid vegetative cells which failed to undergo meiosis. Or, else, they might originate by 'adjustment' (aufregulierung), i.e. vegetative duplication of the chromosome number, similar to what is assumed to occur in *Ulva* (Föyn, 1934), *Enteromorpha* (Moewus, 1938), *Percursaria* (Kornmann, 1956), and *Chaetomorpha* (Köhler, 1956). No cytological evidence of the 'adjustment' of chromosome number is available in the literature.

A more detailed cytological investigation in *P. stipitata* which might clear the problems here put forward is certainly needed.

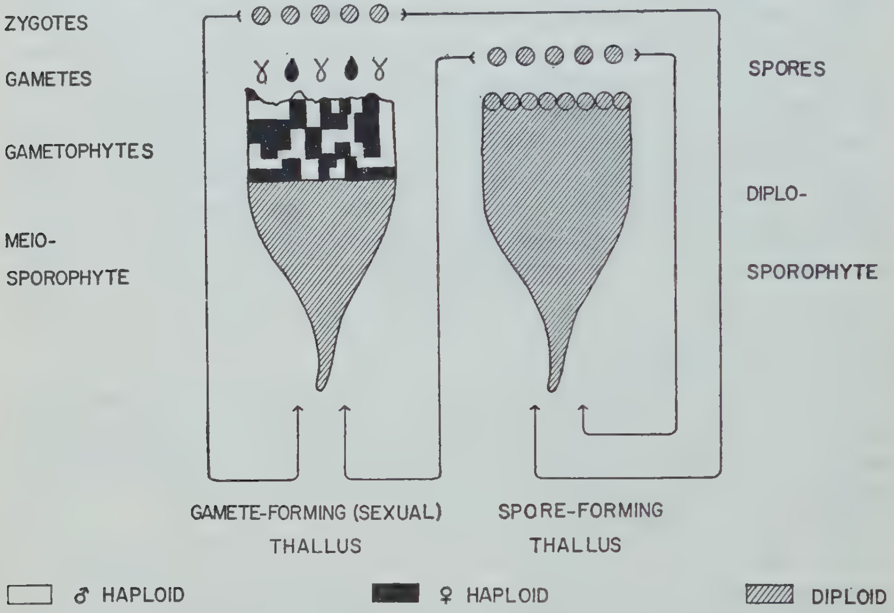
(*f*) *Life-history*

On the evidence provided by the investigations reported in this paper, the life-history of *P. stipitata* can be described as follows:

1. Both spores and zygotes germinate into diploid vegetative thalli. These have the potentiality of developing into either spore-forming or sexual plants.

2. Spore-forming plants are diploid (diplosporophyte) and discharge diploid spores (diplospores).

3. Sexual plants have a complex structure. Their lower section is diploid (meiosporophyte). By reduction division this produces haploid cells which should be regarded homologous to spores (meiospores). These develop into unisexual haploid gametophytes remaining attached to the mother thallus. The upper section of the sexual plants contains a number of gametophytes of both sexes. These produce gametes which subsequently fuse.



TEXT-FIGURE 6. *Prasiola stipitata* Suhr, diagram of the life-history. Spore formation by gamete-forming thalli as well as the filamentous stage (*Rosenvingiella*) which is probably also a part of the life-history, are omitted.

4. The onset of meiotic divisions is determined by external factors which thus control the occurrence in time (seasonality) and distribution in space (zonation) of spore-forming or gamete-forming (sexual) thalli.

5. Sexual plants may produce exceptional diploid spores, apparently in response to the influence of unfavourable external factors.

6. The life-history probably includes a filamentous stage (*Rosenvingiella*) which develops from diplospores.

The scheme in Text-fig. 6 shows the main lines of the life-history. Exceptional spore formation by sexual plants and the filamentous stage are omitted.

P. stipitata thus can be designated as a dimorphic diplohaplont. Its life-history is of a type hitherto not recorded in algae. In order to express it satisfactorily by the symbols of Drew (1955) it is proposed here that brackets should be added to these symbols. The brackets indicate the connexion between the meiosporophyte and the gametophytes and express the fact that these two phases form a single morphological and physiological unit. Accordingly, the life-history of *P. stipitata* can be expressed as follows:

$$(B-a)-B-B,$$

where a stands for the haploid gametophyte and B for the diploid sporophyte.

(g) *A note on taxonomy*

In a paper on the motile gametes (Drew and Friedmann, 1957) doubts were expressed concerning the validity of the name *Prasiola stipitata*, because in case this species would prove to be identical with *P. calophylla* (Carmich.) Menegh., the latter specific name should have priority. Unfortunately, the type specimen of *P. calophylla* could not be found in the herbaria where Carmichael's material could possibly have been kept, i.e. the Royal Botanic Gardens, Kew, The British Museum (Natural History), London, and the Royal Botanic Garden, Edinburgh. In the Kew Herbarium, however, a specimen was found which Miss Carola Dickinson, in a letter to the author, believes to be that of Carmichael, although it bears neither date nor locality. As this specimen agrees well with the figures in Greville (1826) and as, moreover, it might be the only existing specimen of Carmichael, it should perhaps be designated as a lectotype. The specimen contains only young, vegetative thalli but it shows a characteristic cell pattern with thick longitudinal walls which is clearly different from young vegetative thalli of *P. stipitata* (Plate 3, Figs. 6–8). It seems, therefore, that the specific name *Prasiola stipitata* Suhr should be regarded as valid.

ACKNOWLEDGEMENTS

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The investigation reported in this paper was carried out in the Department of Cryptogamic Botany, University of Manchester, England (October 1956—February 1958) and in the Department of Botany, The Hebrew University, Jerusalem, Israel (March–November 1958).

SUMMARY

1. *Prasiola stipitata* has a double cell wall. The cells possess an individual cellulose membrane and the whole thallus is covered by a coating lamella. This latter has a role in spore and gamete discharge.

2. The chromatophores show a considerable variation in shape and position.

3. Vegetative thalli are one-layered. Owing to the polarity of the cells they are dorsiventrally symmetrical. They are diploid and possess the potentiality to develop into spore-forming or gamete-forming (sexual) thalli.

4. Spore-forming thalli produce diploid spores by periclinal divisions. There are two types of spore formation, the marginal and the variegated type.

5. Sexual thalli are composed of two parts. The lower is diploid and similar to the corresponding portion of the spore-forming plant. This produces by meiotic divisions haploid cells which are regarded as meiospores. These develop into haploid multilayered male and female gametophytes which remain attached to the mother thallus, arranged in a characteristic patchwork pattern.

6. Occasionally, diploid spores appear in haploid gametophytic tissues.

7. In culture, spores were observed to germinate into diploid sporophytes. The appearance of the marginal or variegated type of spore formation was controlled by the composition of the culture medium. Zygotes, as well as spores produced by haploid (gametophytic) tissues, germinated also into diploid thalli. The filamentous stage (*Rosenvingiella*), presumably a stage in the life-history of *Prasiola*, appeared in two cultures of germinating spores.

8. Diploid and haploid mitoses are similar. However, diploid mitoses in periclinal spore-forming divisions differ from the regular pattern. Meiotic division is described. The haploid or diploid status of cells can be determined, without counting the very small chromosomes, by the difference in size of the chromatine mass in ana- and telophase.

9. The chromosome number is 6 (7) in haploid and 12 (14) in diploid cells. Occasionally, cells with at least 10–12 chromosomes appeared in haploid tissues.

10. Problems concerning the homology in spore-forming and sexual thalli are discussed.

11. Sex determination is haplogenotypic. The onset of meiosis and thus the formation of sexual thalli is controlled by external factors.

12. The life-history is of a dimorphic diplohaplontic type.

13. The validity of the species *P. stipitata* Suhr is confirmed.

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APPENDIX

THE brazilin staining method used in these investigations was initiated by Dr. K. M. Drew-Baker. As far as could be established, this was never published by her and so it is communicated here.

Brazilin for staining algal whole mounts

Fixation. Remove excess moisture from material and plunge into:

70 per cent. alcohol	100 c.c.
40 per cent. formaldehyde	6 c.c.

Shake well. After 24–48 hours (or longer) pour off used fixative and replace by fresh. Place material in strong light to bleach.

Staining: 1. Wash in 70 per cent. alcohol.

2. Mordant for 1 hour in alcoholic alum:

4 per cent. aqueous iron alum	23 c.c.
90 per cent. alcohol	77 c.c.

For many algae this solution should not be used until 4–6 weeks after being made.

3. Wash for 1 hour in several changes of 70 per cent. alcohol.

4. Stain in 0·5 per cent. brazilin in 70 per cent. alcohol, for 18–48 hours. This solution takes 4–6 weeks to ripen and it is convenient to have a concentrated stock solution from which dilutions can be made.

5. Wash in 70 per cent. alcohol until no excess stain comes out. The time necessary in the following solution varies somewhat according to the material, but those given form a good basis for experiment.

6. Pass through a series of 5 per cent. grades of alcohol, leaving material in each for 15–20 minutes.

7. Place in absolute alcohol for 1 hour, changing liquid once.

8. Pass through series of seven grades absolute alcohol-xylol mixtures, leaving material 15–20 minutes in each.

9. Place in xylol for 1 hour.

10. Place in dilute balsam-xylol mixture and leave for several hours (overnight is often convenient).

11. Pass through three higher-grade balsam-xylol mixtures and mount.

The depth of the stain depends on the strengths of the mordant (which in turn depends partly on the strengths of the initial solution and partly on its age) and also on the length of mordanting time.

Lit.: Popham, R. A.: Mordanting Plant Tissues. Stain Techn. **23**, No. 2, pp. 49–54, 1948.

EXPLANATION OF PLATES

Plate 1

Prasiola stipitata Suhr, photographs of living thalli viewed from the surface. 1: Cells of the basal region. 2: Vegetative cells (focus on the chromatophores and the pyrenoids). 3: Vegetative cells close to the basal region. Here cell arrangement is somewhat irregular. (Focus on the chromatophores and the pyrenoids.) 4: The same cells as in Fig. 3, with focus on the nuclei. The lobes of the chromatophores are visible as dark spots. 5: Vegetative cells (focus on the nuclei). 6: Spore formation (cells as in Text-fig. 1B). 7: Spores after discharge. 8: Young gametophytic tissue, male and female cells.

Plate 2

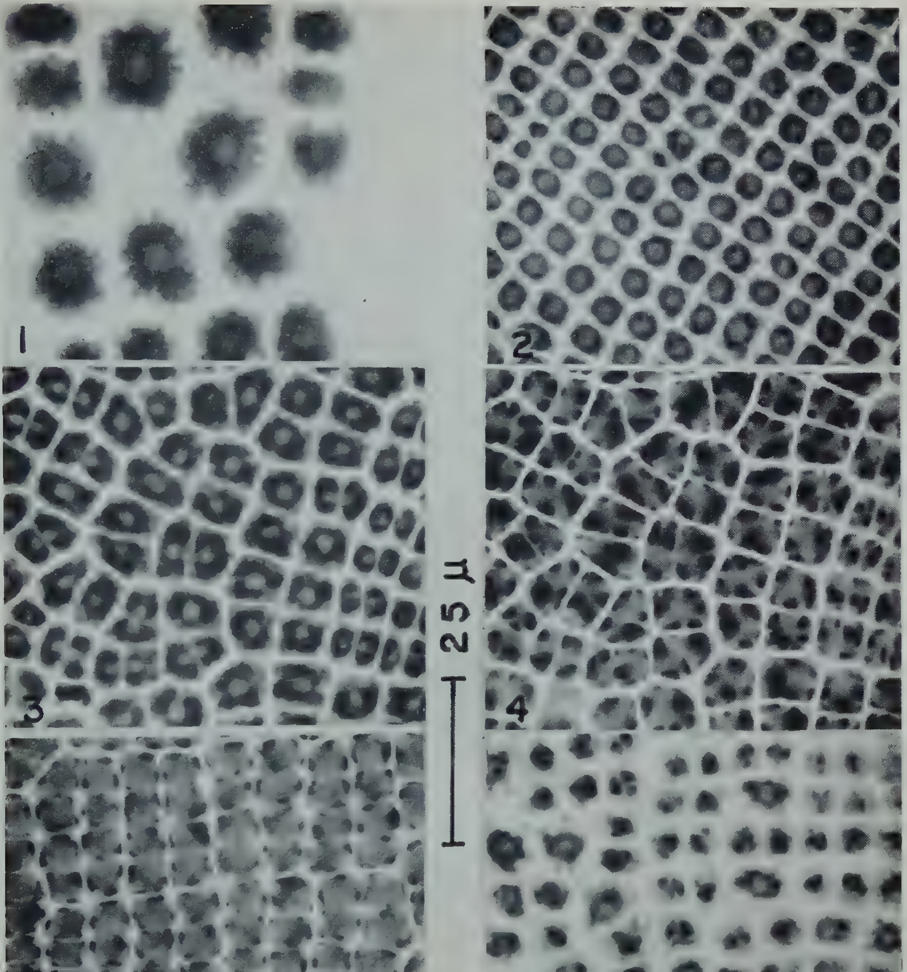
Prasiola stipitata Suhr, photographs of living thalli. 1, 2: Cells viewed from the surface of the thallus. 1: Mature gametophytic tissue, male and female cells. 2: Spores in a gamete-forming thallus. The mature gametophytes have partly discharged the gametes. Between the gametophytic cells there are large spores. 3: Gamete-forming thallus. 4: Apical region of a gamete-forming thallus, showing the difference in the size of the chromatophores in male and female cells. 5: Apical region of a gamete-forming thallus after the discharge of gametes. The zygotes remain in the persisting bladder-like coating lamella. 6: Apical region of a gamete-forming thallus. 7: Apical region of a young gamete-forming thallus. 8: The same as in the previous figure. The inked lines mark the single pairs of male and female gametophytes, each pair resulting from a separate meiotic division.

Plate 3

Prasiola stipitata Suhr (Figs. 1–7) and *Prasiola calophylla* (Carmich.) Menegh. (Fig. 8). Figs. 1–5 are photographs of living thalli. 1: Spore-forming thallus of the marginal type. 2: Apical portion of a spore-forming thallus of the marginal type with discharged spores. 3: Part of a spore-forming thallus of the variegated type shown in the next figure. 4: Spore-forming thallus of the variegated type. 5: Decaying vegetative thallus collected at the end of the growing season, June 21, 1957, at Beaumaris. Between the dead cells there persist several cells with chromatophores like those of spores. 6 and 7: Herbarium specimens of young vegetative thalli. 6: Collected December 12, 1957, at Beaumaris. 7: Collected January 16, 1958, at Beaumaris. 8: *Prasiola calophylla*, Carmichael's specimen in Kew Herbarium, showing thick longitudinal membranes.

PLATE 4

Cytology of *Prasiola stipitata* Suhr, photographs of whole mounts. Figs. 7 and 8 are stained with brazilin, the rest with aceto-carmine. 1: Diploid anatelophase and interphase in a young vegetative thallus. 2: Diploid late prophase with 12–14 chromosomes in a young vegetative thallus, germinated in culture from a spore formed by a gamete-forming plant. 3: Diploid (?) cell in a haploid gametophyte, late prophase with at least 10–12 chromosomes. Neighbouring cells showed the normal haploid chromosome number. 4: Meiotic zone in a young gamete-forming thallus. The top cell is in first meiotic ana-telophase, the left bottom cell is in second meiotic ana-telophase. The oblique division on the top is irregular. 5: Haploid mitotic anaphase and metaphase in male cell, spindle view. 6: Haploid mitotic anatelophase and metaphase in female cells, spindle view. 7: Haploid late prophase. 8: Haploid metaphase plate. 9: Diploid late prophase in transversal spore-forming division. The nucleus lies beside the chromatophore which is unstained. 10: Diploid resting nucleus. 11–15: Stages in meiotic prophase. 11: Early prophase. 12: Later prophase. 13: Pachytene. 14: Late diplotene. 15: End of diakinesis, cf. Text-fig. 5. 16: Division of the pyrenoid. 17: Meiotic metaphase in spindle view. A single pair of univalents separating precociously. 18–19: Meiotic anaphases in spindle view. A single pair of univalents in precocious separation.



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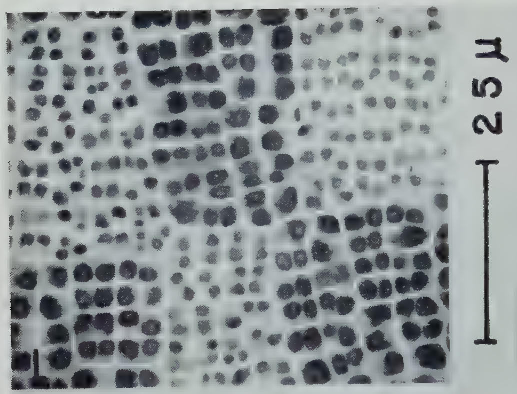
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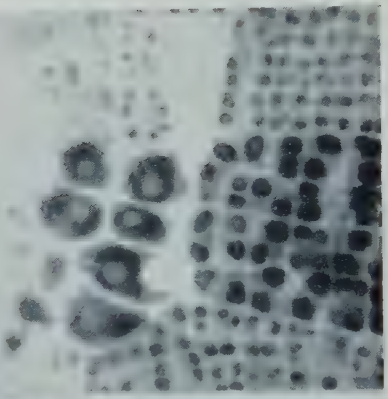
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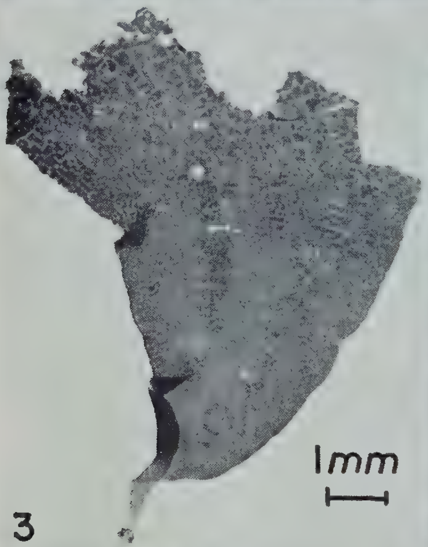
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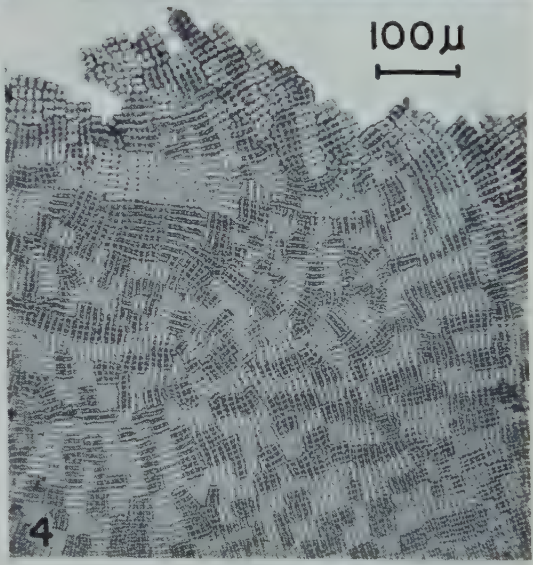
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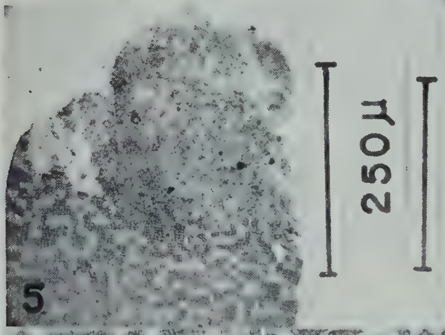
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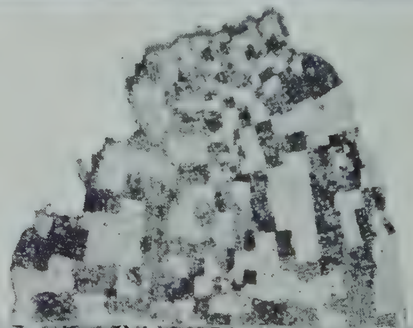
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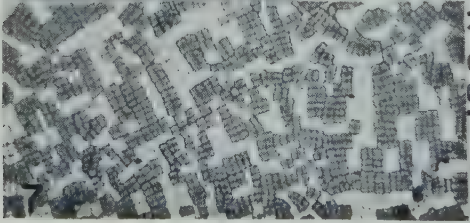
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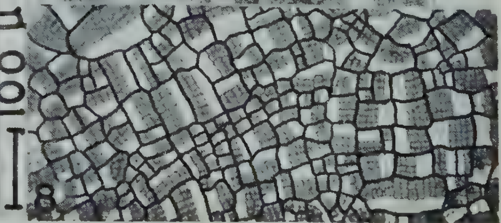
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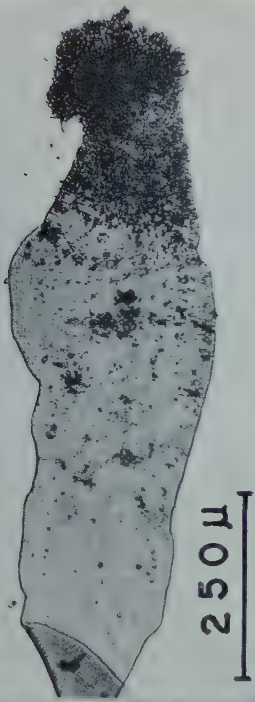

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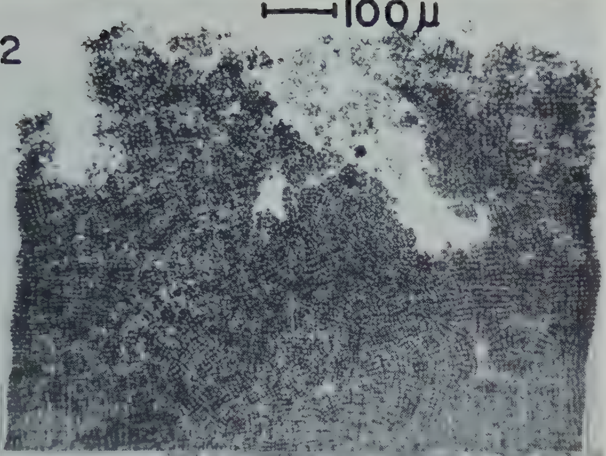

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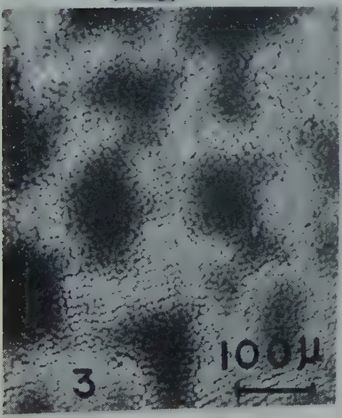

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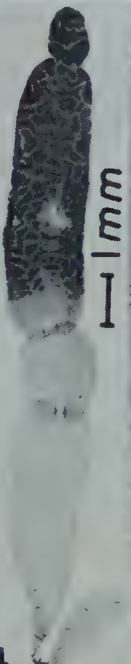

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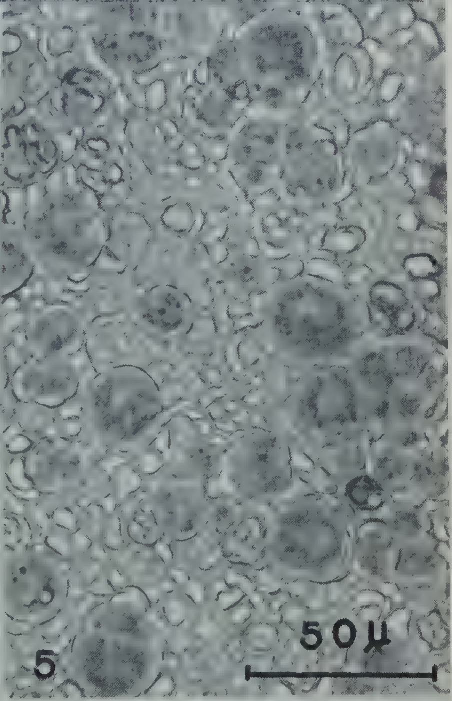

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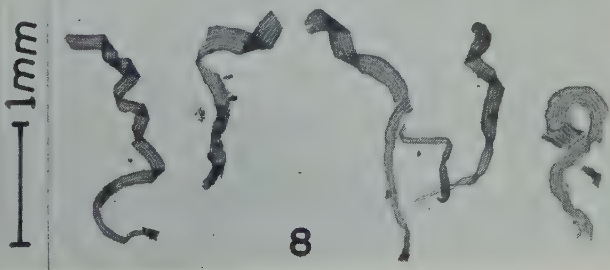

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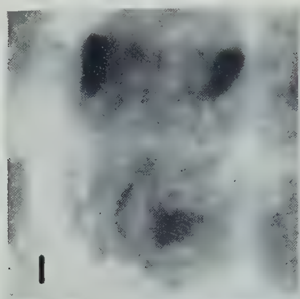
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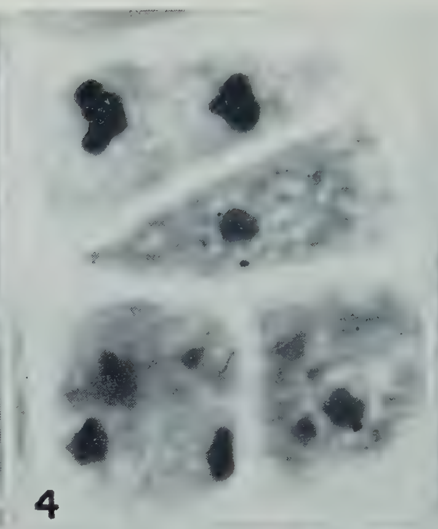
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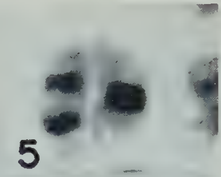
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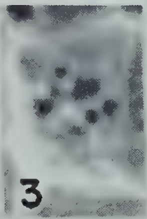
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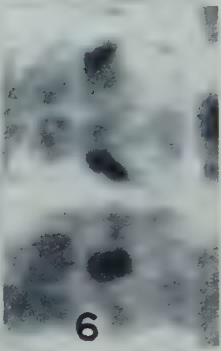
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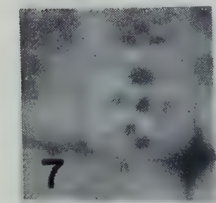
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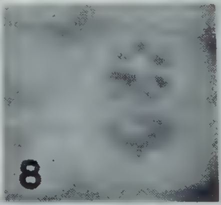
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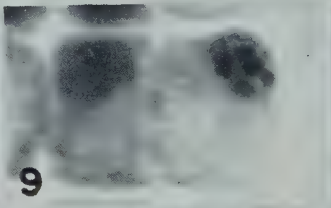
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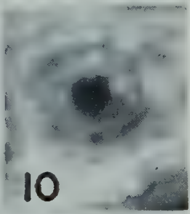
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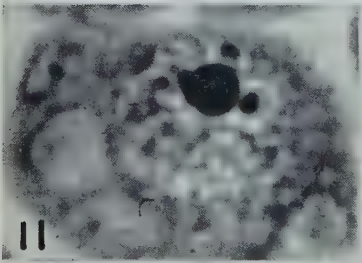
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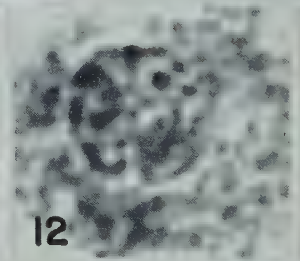
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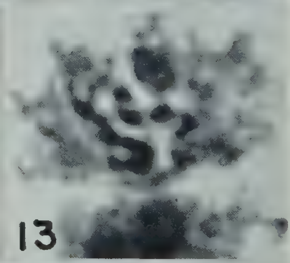
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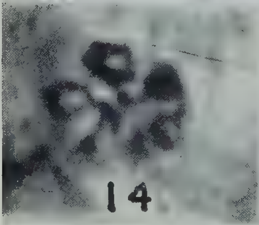
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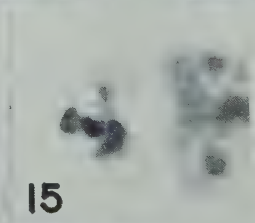
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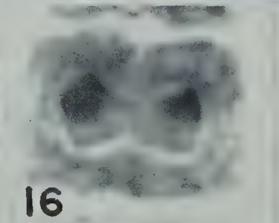
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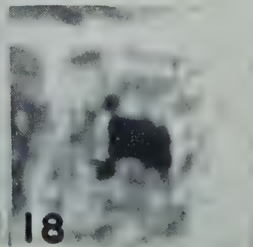
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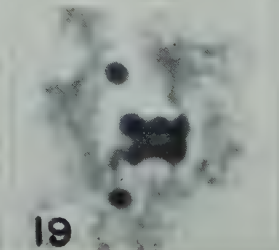
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I. FRIEDMANN

Experiments with Spores of Aquatic Hyphomycetes

I. Sedimentation, and Impaction on Smooth Surfaces

BY

JOHN WEBSTER

(*Botany Department, The University, Sheffield 10*)

With seven Figures in the Text

Abstract

Techniques are described for the production of heavy concentrations of aquatic Hyphomycete spores, for estimating the spore concentration, the sedimentation rate of spores and their trapping efficiency on smooth cylinders. No correlation could be discovered between the rates of sedimentation and spore shape. Trapping experiments showed that the trapping efficiency of the tetraradiate spores was higher than that of spores of different shape. It is suggested that the higher efficiency might be the result of contact of the tetraradiate spore at three points on a surface.

INGOLD (1942, 1943*a*, 1943*b*, 1943*c*, 1944, 1949, 1952, 1956, 1958*a*, 1958*b*) and his collaborators (Ingold and Ellis, 1952; Ingold and Cox, 1957*a*, 1957*b*) have described a remarkable flora of fungi growing on decaying leaves of deciduous trees in rapidly flowing streams. A large number of species has been described and many more already recognized as spores in foam from the surface of streams await description until they are found attached to their substrata so that details of spore development can be followed. The mycelia of these fungi grow within the tissues of the decaying leaves and produce conidiophores which project into the water and bear spores. The spores vary in shape, being subspherical in *Margaritispora aquatica*, cylindrical in *Piricularia submersa*, crescent-shaped in *Lunulospora curvula* and sigmoid in *Flagellospora* spp. and *Anguillospora* spp. In many of the species the spore consists of four (or occasionally more) arms radiating from a central point or as branches from a main axis. Ingold's painstaking and elegant studies on spore development in these tetraradiate forms have shown that the spore may be formed in a number of distinct ways. For example, in *Lemonniera*, *Actinospora* and *Heliscus* the spores arise from phialides, whilst in *Articulospora*, *Tetracladium*, and *Tricladium* the spores are terminal thallospores. Further criteria separating the genera are the method of origin of the arms (in certain forms the arms arise simultaneously, in others successively) and the presence of knoblike appendages (e.g. *Tetracladium*). Ingold (unpublished) has also noted a tropical form bearing clamp-connexions at the septa. This evidence suggests that the forms with tetraradiate spores may be phylogenetically diverse; that convergent evolution has resulted in the formation of spores of tetraradiate form in unrelated fungi. Although there are occasional records of some of these fungi

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from terrestrial habitats (Bessey (1939), Scourfield (1940), Waid (1954)) there can be no question that they are abundant on leaves in streams throughout the world (Ingold (1943*c*, 1949), Fenton (1950), Ranzoni (1951), Tubaki (1957), Nilsson (1958)). In a number of species other forms of spore are known. For example, *Flagellospora penicillioides* and *Heliscus lugdunensis* (syn. *H. aquaticus*) both forming phialospores, are known to be the conidial states of species of *Nectria* known so far only in culture (Ranzoni (1956), Webster (1959)). *Clavariopsis aquatica* forms pycnospores in culture (Ingold, 1942) and *Heliscus longibrachiatus* forms 'monilioid' arthrospores in culture. Whether such spores are formed and dispersed from leaves in streams is not known.

In addition to these freshwater fungi a number of marine fungi have tetraradiate spores, e.g. *Orbimyces spectabilis* (Barghoorn and Linder, 1944). The propagule of the brown alga *Sphacelaria* as noted by Ingold (1953) forms a striking parallel in form to the propagule of many aquatic hyphomycetes. The sigmoid form of spore is also characteristic of other aquatic organisms, e.g. some ascomycetes from freshwater (Ingold, 1954) and from seawater (Barghoorn and Linder (1944), Wilson (1954)).

The fact that the tetraradiate type of propagule has been found in a number of unrelated aquatic organisms has led to speculation about the significance of such structures. Ingold (1953) has written

> . . . in aquatic Hyphomycetes the tetraradiate spore is of such frequent occurrence that it is natural to suppose it has some biological significance in the aquatic environment. What this significance may be is pure speculation. Perhaps a spore of this kind settles relatively slowly in water and so stands a good chance of adequate dispersal; perhaps on the other hand it acts as an anchor and readily becomes entangled in a suitable substratum, for the arrest of a spore in a stream may be a real problem; or perhaps it is not so easily devoured by small aquatic animals as a spherical or oval spore would be.

It has recently become possible to examine the first two speculations experimentally because of advances in technique. Firstly, it was discovered that forced aeration of cultures of aquatic hyphomycetes stimulates sporulation so that heavy spore concentrations of the order of 30,000/ml. could be produced. Secondly, it was discovered by Mr. T. L. C. Bottomley that the spores of aquatic hyphomycetes are arrested by cellophane-coated rods placed in streams. Modification of this method for use in the laboratory has permitted the study of spore deposition at different water velocities.

TECHNIQUES

(*a*) *Production of spores.* Pure cultures of aquatic hyphomycetes were started from single spores. Leaves collected from streams in the vicinity of Sheffield were kept in the laboratory for 2–3 days in shallow dishes of water. Sporulation of aquatic hyphomycetes took place and spores were picked off by hand from the surface of the water, using a fine sewing-needle or from suspension by a fine capillary pipette. Spores were planted on 2 per cent. malt agar to which antibiotic had previously been added, following the amounts recommended by Vishniac (1956), and after a few days transfers were made. Bacteria-free cultures were usually established with little difficulty. In addition to these isolates we are indebted to Professor Ingold, Dr. Ranzoni, Dr. Tubaki, Dr. Haskins, and the Centraalbureau voor Schimmelcultures for others. Spores of the fungi used in the experiments are illustrated in Fig. 1.

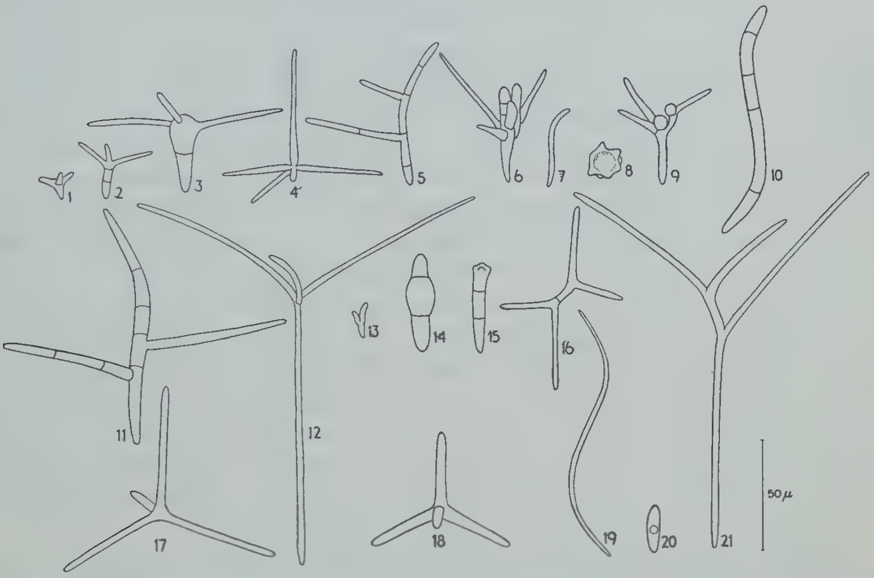

FIG. 1. Spores of aquatic hyphomycetes used in experiments, drawn to a uniform magnification. Spores 1–16 are arranged in the order of sedimentation rates tabulated in Table 1. 1. *Heliscus stellatus;* 2. *Heliscus longibrachiatus*; 3. *Clavariopsis aquatica*; 4. *Triscelophorus monosporus*; 5. *Varicosporium elodeae*; 6. *Tetracladium setigerum*; 7. *Flagellospora penicillioides*; 8. *Margaritispora aquatica*; 9. *Tetracladium marchalianum*; 10. *Anguillospora longissima*; 11. *Tricladium splendens*; 12. *Tetrachaetum elegans*; 13. *Tricellula aquatica*; 14. *Dactylella aquatica*; 15. *Heliscus lugdunensis*; 16. *Tricladium angulatum*; 17. *Lemonniera aquatica*; 18. *Lemonniera brachycladia;* 19. *Flagellospora curvula*; 20. *Glomerella cingulata* conidia; 21. *Tricladium gracile*.

When cultures were required for sporulation, a portion of a stock culture was macerated in an M.S.E. homogenizer for a few seconds and the mycelial fragments so obtained were added to 2 per cent. malt extract agar previously melted and cooled to 45° C. Plates were poured from the inoculated media and incubated at room temperature in diffuse light for 1 week or more. In some cases, e.g. *Articulospora tetracladia*, sporulation could be induced in 1-week-old cultures, but in others, e.g. *Margaritispora aquatica*, longer periods of growth were required.

Spores were obtained by cutting up the cultures into pieces of approximately 1 square centimetre. These pieces were transferred to screw-top bottles containing about 1 litre of sterile tap-water. Compressed air was forced through the water, thus causing continuous agitation of the culture. A convenient method of producing a large number of small air bubbles was to aerate the culture through silicone-rubber tubing, sealed at one end and punctured at intervals along its length by a needle. Sporulation took place within 48 hours and could be detected readily in the case of the tetraradiate forms by a deposit of spores formed on the glass above the water line. So long as aeration was continued such spores did not germinate, and spores were usually harvested 2–3 days after the start of forced aeration. When required, the spore deposit was shaken down into suspension and the spores were separated from the culture pieces and from mycelial fragments by passing through a fine sieve which allowed the spores to pass through. The spore concentration was determined by drawing up a small volume into an 'Agla' Micrometer Syringe (Burroughs Wellcome & Co., London). Ten small drops each containing 0·001 ml. of suspension were then placed on a marked microscope slide. After the drops had dried out the number of spores in each drop was counted. Provided that precautions are taken to mix the spore suspension thoroughly and to prevent sedimentation, spore counts could be made with good replication. The accuracy of this method has been compared with a sedimentation technique. Five ml. of a spore suspension was pipetted into a 6-cm. Petri-dish and the spores allowed to settle overnight. The numbers of spores adhering to the bottom of the dish were counted in 20 microscope fields, and by simple proportion the numbers present on the whole area of the Petri-dish bottom were estimated, and hence the concentration. Comparison with the pipette count showed that the results were often closely comparable, but there was a tendency for the pipette counts to give higher results. This may be due to spore attachment on the *sides* of the Petri-dish.

(*b*) *Sedimentation rates.* The rates of sedimentation were determined in narrow glass tubes (6-mm. internal diameter), closed at the lower end and mounted vertically on a microscope tilted horizontally. Using a previously calibrated eyepiece micrometer and a 16-mm. objective, spores were timed with a stop-watch as they fell through a 1-mm. interval. The rate of sedimentation varies with the distance of the spore from the wall of the tube; spores close to the wall move more slowly than spores some distance away. At low temperatures the rate of sedimentation is decreased. Fig. 2 shows the effect of these factors on the rate of sedimentation of spores of *Articulospora tetracladia*. As a standard for comparison we have worked with spores at a *focal plane* 1 mm. inside the wall of the tube (i.e. at a real distance of 1·33 mm.) and at a temperature of 15° C. obtained in a cooled constant-temperature room. In spite of attempts to maintain constant-temperature conditions it is extremely difficult to prevent convection currents in the sedimentation tubes, and it is certain that this difficulty has not been completely overcome. The results obtained by this method are presented in Table 1.

(*c*) *Trapping.* The apparatus used for investigating the trapping of aquatic hyphomycete spores is shown in Fig. 3. It consists of a reservoir (capacity 15 litres) connected to a Stuart Centrifugal Pump which pumps the water to a 'constant head'. The overflow from the constant head is returned to the reservoir. The main outlet from the constant head leads to the trapping chamber, a glass tube about 120 cm. long and 2·5 cm. internal diameter. The outflow from the trapping chamber is also returned to the reservoir. The rates of flow of water through the trapping chamber can be varied by altering the height of the constant head, or by restricting the outflow from the trapping chamber. The velocity of water passing through the trapping chamber was determined for a given height of the constant head by timing Indian ink jets over 1 m. intervals. The volume of water passing through at given velocities was also determined using a measuring cylinder and a stop-watch. Velocities in the range 0–45 cm. sec. could be obtained with the apparatus. Within this

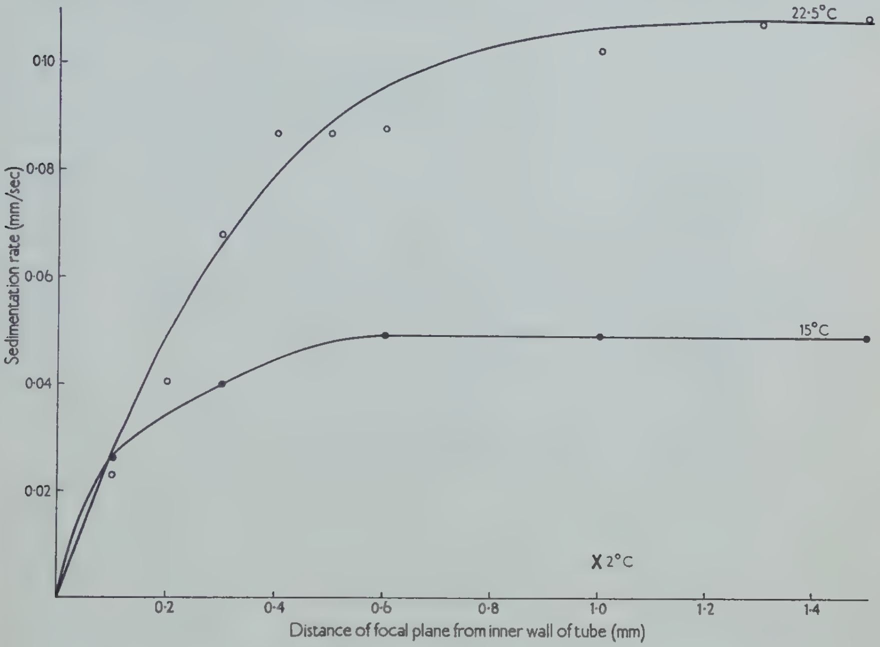

FIG. 2. Rates of sedimentation of spores of *Articulospora tetracladia* at different distances from the inner wall of a vertical glass tube at different temperatures.

TABLE 1

Sedimentation Rates of Aquatic Hyphomycete Spores. Mean Values (mm./sec.) for 20 Spores in Water at 15° C. and at a Focal Plane 1 mm. from the Inner Wall of a Glass Tube

1. *Heliscus stellatus*	0·187±0·0020	9. *Tetracladium marchalianum*	0·086±0·0080
2. *Heliscus longibrachiatus*	0·185±0·0016	10. *Anguillospora longissima*	0·081±0·0075
3. *Clavariopsis aquatica*	0·144±0·0010	11. *Tricladium splendens*	0·064±0·0080
4. *Triscelophorus monosporus*	0·142±0·0036	12. *Tetrachaetum elegans*	0·064±0·096
5. *Varicosporium elodeae*	0·134±0·025	13. *Tricellula aquatica*	0·064±0·0124
6. *Tetracladium setigerum*	0·128±0·0030	14. *Dactylella aquatica*	0·057±0·0058
7. *Flagellospora penicillioides*	0·095±0·0050	15. *Heliscus lugdunensis*	0·052±0·0058
8. *Margaritispora aquatica*	0·087±0·0009	16. *Tricladium angulatum*	0·046±0·0096

range of velocities water flow was streamlined up to 15 cm./sec., but at higher speeds it was turbulent.

A suspension of spores of the fungus under investigation was first counted and then a measured volume of the suspension was added to the reservoir. The total number of spores added was usually adjusted to give a final concentration of spores in the whole apparatus of 100–200/ml. After allowing the pump to run for 2 min. in order to mix the suspension thoroughly, a trap was inserted and left in the apparatus for 5 min.

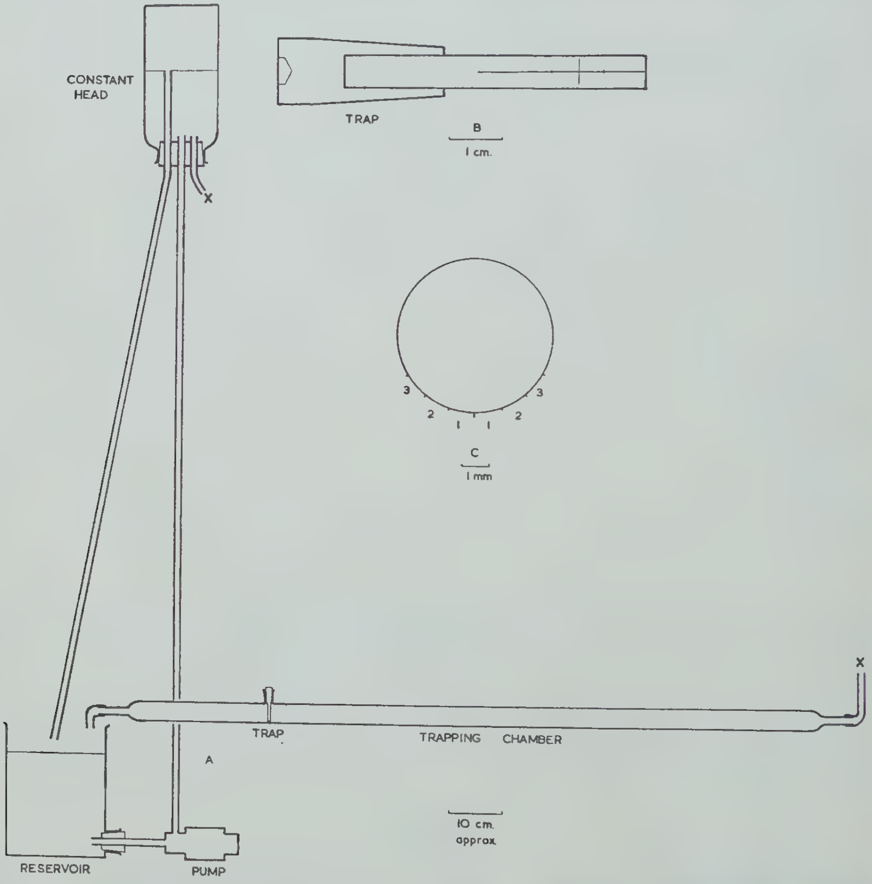

FIG. 3. A. Diagram of trapping apparatus. For explanation see text. B. Diagram of trap showing reference lines. C. Section of trap showing position of the rows of microscope fields on either side of the centre-line.

The trap consists of a length of glass rod 6·5 mm. in diameter, marked along the length with a diamond scratch and with another mark running round the trap 1·2 cm. from one end, the crossing of the two scratches thus marking the mid-point of the trapping chamber. The glass rod is cemented in a Perspex holder turned to fit a Quickfit and Quartz B_{12} socket which is sealed near the distal end of the trapping chamber. The Perspex holder is so marked that the orientation of the longitudinal scratch on the rod can be determined when the trap is in position, and the trap is placed so that the longitudinal scratch points 'upstream'.

The surface of the trap was coated before use with a layer of collodion (Necol Collodion Solution 301–261 (356/A9) from British Drug Houses Ltd.) dissolved in ether. After evaporation of the ether a thin transparent film was left on the trap surface, and this could easily be stripped off before re-coating the traps for the next experiment.

Spores were impacted on the upstream face of the trap, and the numbers were counted. The trap in its mount was placed in a specially constructed holder and held in the mechanical stage of a microscope. A convenient eyepiece-objective combination was chosen, giving a microscope field of 1 mm. diameter. Using the mechanical stage of the microscope, longitudinal strips of the trap surface were scanned for spores. Six rows were scanned, three on either side of the longitudinal mark. Each row consisted of ten microscope fields, five on either side of the transverse mark on the trap. The position of these rows of microscope fields is indicated in Fig. 3, C which shows a section of the trap and the positions of the various rows on the trap. In most of the experiments a comparison was made between the spore counts in the two rows on either side of the longitudinal line, but in others deposition in adjacent rows has also been considered. From these data it is possible to obtain an estimate of the trapping efficiency (see Gregory, 1951) of a particular trap at a given speed. It was found when experiments were repeated that the numerical values for trapping efficiency were not always in close agreement. For this reason all trapping experiments have been repeated twice, and the mean trapping efficiency from the three experiments has been tabulated in Table 2.

RESULTS

1. *Sedimentation rates.* It cannot be doubted that a tetraradiate spore would settle more slowly in water than a spore of spherical shape of equal volume and density, and in the absence of data for cytoplasmic density in different aquatic fungi the problem can best be tackled by considering whether tetraradiate spores as a group settle consistently more slowly than spores of other shape. The results of observations on sedimentation in a series of aquatic hyphomycetes are given in Table 1. The fungi are tabulated in order of observed sedimentation rate. Comparison with the figures of the spores in Fig. 1 shows that the correlation between the rate of settling and the shape of the spore is not particularly good, and that the tetraradiate spore forms as a group do not settle consistently more slowly than spores of other shape. It is possible, however, that with more refined techniques for limiting convection currents within the observation vessel, a closer correlation between spore shape and sedimentation rates might be discovered. The actual rates of settling are all of the order of 0·1 mm./sec. (or less than 1 inch in 4 min.). It is profitable to compare this rate with the rate of flow of streams in which such

TABLE 2

Trapping Efficiency (per cent.) for Various Aquatic Hyphomycete Spores at Five Water Speeds (Mean Values from Three Experiments)

Calculations based on spore deposits in row 1 only

SPECIES	5 cm./sec.	15 cm./sec.	25 cm./sec.	35 cm./sec.	45 cm./sec.	MEAN OF ALL OBSERVATIONS
1. *Tetrachaetum elegans*	12·8±11·05	3·94±1·56	2·69±1·09	2·1±1·22	1·31±0·25	4·56
2. *Articulospora tetracladia*	0·9±0·13	0·35±0·002	0·51±0·002	0·33±0·001	0·30±0·021	0·477
3. *Tricladium splendens*	0·69±0·28	0·32±0·06	0·39±0·28	0·21±0·13	0·10±0·07	0·344
4. *Tetracladium marchalianum*	0·63±0·22	0·28±0·024	0·37±0·029	0·27±0·02	0·15±0·002	0·339
5. *Clavariopsis aquatica*	0·23±0·28	0·3±0·17	0·4±0·33	0·3±0·15	0·28±0·24	0·301
6. *Lemonniera brachycladia*	0·19±0·05	0·27±0·06	0·46±0·05	0·36±0·08	0·18±0·03	0·293
7. *Tetracladium setigerum*	0·56±0·11	0·24±0·022	0·30±0·04	0·19±0·04	0·12±0·05	0·281
8. *Lemonniera aquatica*	0·61±0·45	0·35±0·09	0·13±0·033	0·04±0·014	0·039±0·18	0·232
9. *Tricladium gracile*	0·32±0·15	0·39±0·14	0·23±0·01	0·1±0·05	0·07±0·13	0·222
10. *Varicosporium elodeae*	0·14±0·1	0·16±0·05	0·15±0·06	0·12±0·05	0·1±0·04	0·133
11. *Anguillospora longissima*	0·1±0·066	0·11±0·07	0·1±0·06	0·1±0·04	0·06±0·01	0·096
12. *Flagellospora curvula*	0·049±0·03	0·06±0·026	0·136±0·07	0·14±0·05	0·08±0·054	0·095
13. *Flagellospora penicillioides*	0·085±0·10	0·023±0·016	0·023±0·01	0·013±0·09	0·063±0·002	0·023
14. *Heliscus lugdunensis*	0·01±0·02	0·004±0·005	0·009±0·008	0·007±0·003	0·006±0·003	0·008
15. *Dactylella aquatica*	0	0·004±0·002	0·009±0·036	0·008±0·0002	0·007±0·002	0·006
16. *Glomerella cingulata* conidia	0·0013±0·002	0·0006±0·009	0·002±0·003	0·0012±0·005	0·0023±0·002	0·0025
17. *Tricellula aquatica*	0·0006±0·00009	0·0023±0·004	0·0026±0·001	0·0029±0·001	0·0032±0·0027	0·0024

fungi abound. A series of rough determinations of current speed were made by timing match-sticks over 1 m. intervals with a stop-watch in a number of small streams near Sheffield. Speeds up to 5 m./sec. were encountered (higher speeds were also found but were difficult to estimate by this technique), but the majority of determinations were of the order of 1 m./sec. If the path of a spore is considered as a parallelogram of velocities with a vertical component due to sedimentation of the order of 0·1 mm./sec. and a horizontal component due to stream flow of the order of 1,000 mm./sec. it is clear that the rate of sedimentation is of negligible proportion.

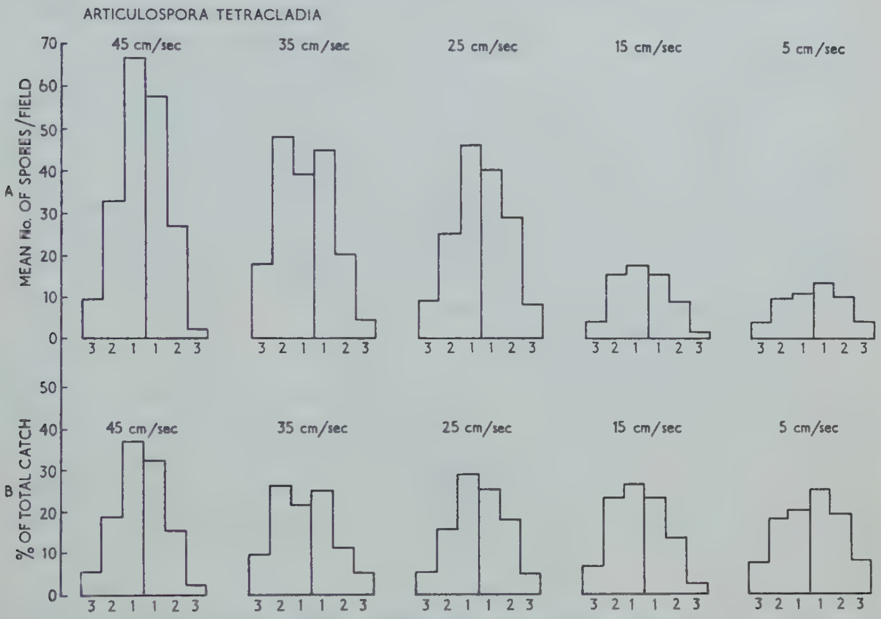

FIG. 4. The pattern of deposition of spores of *Articulospora tetracladia* at various speeds. A. Actual counts. B. Counts expressed as a proportion of the total catch.

These studies on sedimentation rates therefore provide no evidence that the tetraradiate spore settles more slowly than spores of more conventional shape, and whilst such spores would undoubtedly remain in suspension and be dispersed by currents, they appear to be no more effective in this respect than spores of different shape. It is difficult to imagine situations apart from lakes and small bodies of water in the non-turbulent condition where sedimentation accounts primarily for the removal of spores from suspension.

2. *Trapping experiments.* The results of a typical trapping experiment are shown in Fig. 4 A, which shows the pattern of deposition of spores of *Articulospora tetracladia* on traps exposed for 5 min. to aliquot spore suspensions at water speeds of 5, 15, 25, 35, and 45 cm./sec. The histograms show that the majority of spores fall on the two rows adjacent to the centre line, and that the spore deposit decreases round the sides of the trap. The numbers caught on

row 4 were negligible. These patterns of deposition parallel those described in wind tunnel experiments using spores of *Lycopodium* by Gregory (1951). If the numbers caught in each row are expressed as a proportion of the total catch (Fig. 4 B) we see that at high speeds the proportion of spores caught near the centre line increases (or, in other words, the spore trace becomes narrower).

In explaining these results it should be borne in mind that the presence of a spore in a given position on a trap is dependent on two processes: deposition and subsequent removal by washing off. It is possible to make a rough estimate of the magnitude of the effect of washing off by placing spores on a series of traps at a uniform speed, counting them and then washing in clean water for 5 min. (the normal duration of an experiment) and then recounting. The results of such an experiment are shown in Fig. 5 B which shows that the proportion of spores washed off is affected by the position of the spore on the trap, the proportion increasing with distance away from the centre-line. The effect of position is more important than velocity since, in the range of velocities investigated, there is no appreciable increase in percentage spore loss with increasing speed of water flow.

These estimates of the spore loss due to wash-off are high since in a normal experiment a spore arriving late on a trap would not be exposed to the effects of wash-off for a full 5 min. This error may be offset by the fact that in making the first count in a wash-off experiment one is counting spores which have already survived a period of washing, and at the second count one is therefore studying the continued survival of the spores during a further wash-off period. The two errors tend to compensate, but it is difficult to assess their relative magnitude. If in spite of this difficulty the estimate is accepted, it can be used to correct the counts in experiments in which spores are deposited at different speeds. Such a correction is illustrated in Fig. 5 C. Even after applying the correction due to estimated wash-off the pattern of spore deposition is approximately the same as before with a denser deposit near the centre-line. It is, therefore, concluded that the physical conditions of water flow at the various positions on the trap are such that the chances of *deposition* on the lateral positions are much lower than in the central positions.

The number of spores caught on the front face of the traps in the experiment illustrated in Fig. 4 A is plotted against speed in Fig. 6, which shows an increasing spore deposit with increasing speed. But at high speeds a greater volume of water, and therefore a larger number of spores, passes over the trap during the duration of an experiment than at low speeds. To interpret the results satisfactorily it is necessary to make a correction for the increasing volume, i.e. to make an estimate of the number of spores caught per litre of suspension. These values are also plotted against current speed in Fig. 6, which shows that the number of spores trapped per litre falls from a higher value at 5 cm./sec. to a more or less consistently low value at higher speeds. It seemed possible that this effect might be due to the fact that at high speeds spores which had been deposited on the trap surface might be washed off again. To test this wash-off experiments were conducted as before, and the percentage loss determined for the various speeds. These results are shown in the inset table in Fig. 6. There is no evidence of a consistently greater wash-off effect at higher speeds, and it is therefore concluded that the reduced trapping efficiency is a real effect due to non-deposition and not due to wash-off.

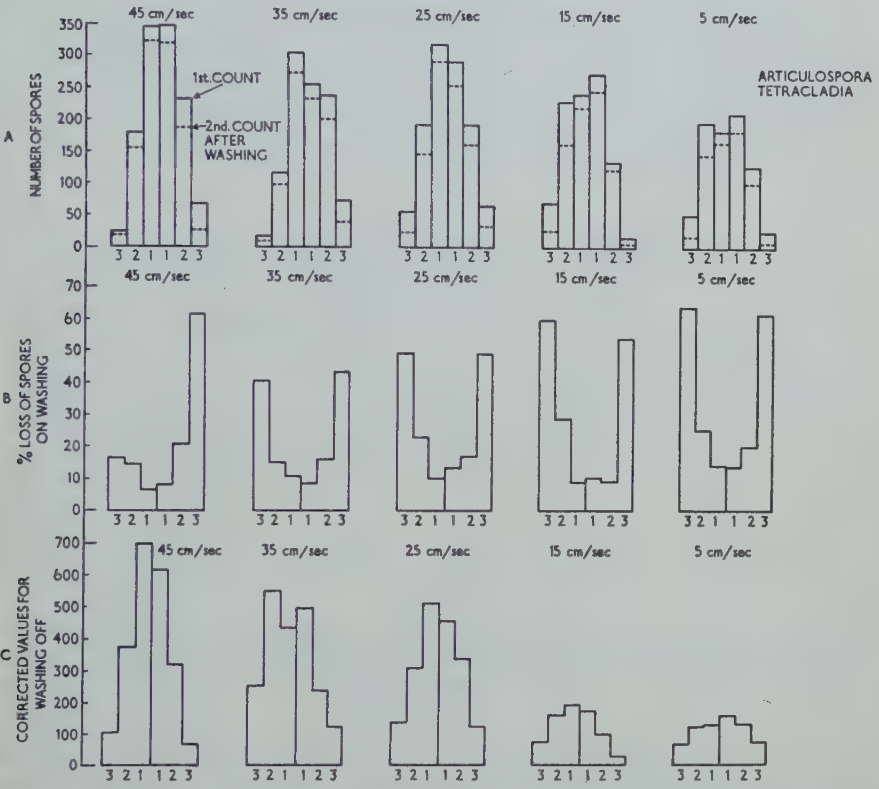

FIG. 5. The effect of washing for 5 min. on deposited spores of *Articulospora tetracladia*. A. Spores deposited at a uniform speed and counted (1st counts). Traps then exposed to washing for 5 min. at the speed indicated and recounted (second counts).
B. Per cent. loss on washing for each row at different speeds.
C. Correction for per cent. wash-off applied to the results illustrated in Fig. 4 A.

Essentially similar results have been obtained for other fungi but in some cases there is evidence of a reduction in *total deposit* of spores at high speeds, for example in *Tetrachaetum elegans* and *Tetracladium marchalianum*, which show maximum deposition in the speed range 25–35 cm./sec. Different patterns were, however, discovered for three of the fungi studied, namely *Tricladium angulatum*, *Varicosporium elodeae*, and *Dactylella aquatica*. Here the curve for number of spores trapped per litre of spore suspension rose from a low value at 5 cm./sec. to a maximum at 25 cm./sec., falling to a low value at higher speeds. The differences in behaviour are at present unexplained.

Another way of expressing these results which allows comparison between

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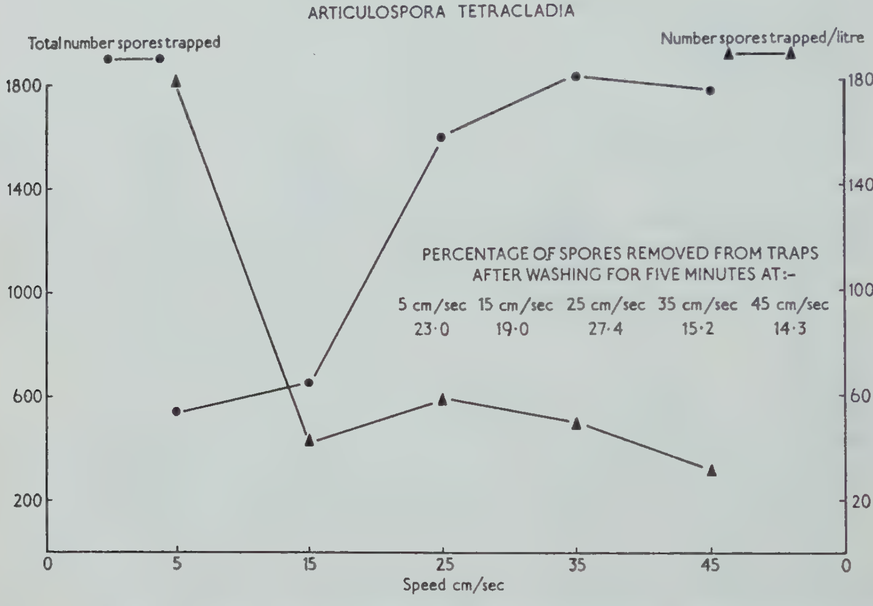
one fungus and another is to use the concept of *trapping efficiency* (see Gregory, 1951). If one considers the cross-sectional area of the trapping chamber it is possible to make an estimate of the number of spores passing through the area provided that the spore concentration and the volume of suspension passed are known, and assuming that spore loss within the apparatus is negligible. (In an experiment designed to discover the spore loss during the course of an experiment three successive 5-minute runs using the same suspension showed a progressive reduction in spore deposit. Expressing the count during the first 5-min. trapping period as 100, the values during the second and third runs were 82·5 and 63·8 respectively. This indicates a loss of the order of 20 per cent. during the course of a 5-min. experiment.) It is therefore possible to estimate how many spores would have passed through the area of a microscope field on the trap surface supposing that the trap had not been in position. The number of spores caught in a given microscope field can thus be expressed as a percentage of the number which should theoretically have passed through it had it not been in place. These percentage values have been calculated for a number of aquatic hyphomycetes (means of three experiments) in the speed range available and are tabulated in Table 2. Since the percentage value varies with speed in a different way for the different fungi, a mean value has been calculated for all the speeds studied, and the list is arranged in order of mean trapping efficiency. The first ten places in the list



PERCENTAGE OF SPORES REMOVED FROM TRAPS AFTER WASHING FOR FIVE MINUTES AT:-

5 cm/sec	15 cm/sec	25 cm/sec	35 cm/sec	45 cm/sec
23·0	19·0	27·4	15·2	14·3

FIG. 6. Numbers of spores of *Articulospora tetracladia* caught on the upstream face of traps from aliquot suspensions at various speeds in 5 min., and the number caught/litre of suspension passing over the traps. The inset table gives an estimate of the effect of washing-off during the 5-min. period.

are taken by tetraradiate forms, followed by the three sigmoid forms examined. Other spore types were relatively inefficient.

The results show that the tetraradiate spore is caught more efficiently on the trap surface than other spore forms studied. A way of confirming this is to use a mixed spore suspension of equal concentration and to count the numbers of spores of different types caught on the trap face. The results can be expressed as a ratio of the numbers of the different spores caught, and this ratio is also a ratio of the trapping efficiencies. A number of such comparison experiments has been done, comparing tetraradiate spores with spores of more conventional shape, and the results of the experiments are given in Table 3.

TABLE 3

Ratio of Numbers of Spores Caught from Equal Concentrations of Mixed Spore Suspensions at Various Speeds

Calculations based on spore deposits in rows 1, 2, and 3.

Species compared	5 cm./sec.	15 cm./sec.	25 cm./sec.	35 cm./sec.	45 cm./sec.
Articulospora tetracladia with:					
Anguillospora longissima	21·7	16·7	22·9	32·0	16·8
Flagellospora penicillioides	3·13	6·58	52·1	33·8	40·7
Heliscus lugdunensis	9·54	10·1	23·0	10·8	30·7
Dactylella aquatica	5·65	97·8	153·0	36·9	51·8
Tetracladium marchalianum with:					
Anguillospora longissima	4·23	1·80	1·74	2·73	3·51
Flagellospora penicillioides	4·70	7·92	12·6	12·5	15·0
Heliscus lugdunensis	11·5	6·33	9·47	11·2	14·8
Dactylella aquatica	∞	1·07	5·99	16·3	21·0
Tetracladium setigerum with:					
Anguillospora longissima	∞	7·23	11·8	8·15	8·06
Flagellospora penicillioides	0·6	16·5	18·3	16·9	7·5
Heliscus lugdunensis	15·9	3·84	5·91	11·7	7·5
Dactylella aquatica	28·5	47·3	78·7	26·1	12·4
Tetrachaetum elegans with:					
Anguillospora longissima	27·0	11·2	22·7	19·2	21·3
Flagellospora penicillioides	∞	44·8	243	50·0	39·9
Heliscus lugdunensis	248	200	1827	5612	62·1
Dactylella aquatica	∞	75·3	8·1	16·8	4·1

In general the tetraradiate spore is more effectively trapped than any other spore type with which it has been compared. An interesting point emerges in the case of *Flagellospora penicillioides.* In most comparisons made the *relative* efficiency for this spore is highest at low speeds, and when compared with *Tetracladium setigerum*, it may actually be more efficient at 5 cm./sec. It will be noted that the ratios given in Table 3 do not conform at all closely with the expected ratios which could be obtained by comparing the trapping efficiency values given in Table 2; the numbers of non-tetraradiate spores are higher than expected. This effect is probably due to the fact that the presence of tetraradiate spores on the trap increases the deposition of other spore types, due to a 'roughening' of the surface. Thus the two types of experiment are not directly comparable. The results of the comparison experiments do, however, confirm the conclusions based on single species trapping experiments, and show that the tetraradiate spore form is caught more efficiently on the trap surfaces than the other spore forms studied.

DISCUSSION

The experiments described above leave no doubt that the tetraradiate spore is caught more efficiently on the trap surfaces than spores of more conventional shape. A probable reason for the higher trapping efficiency is that when such a spore makes contact with a surface it does so at three points, the tips of three arms forming a tripod, a very stable form of attachment. This fact can be demonstrated simply by observing the attitudes of spores which have become detached from leaves in Petri-dishes containing water. The spores sink to the bottom of the dish, and where they make contact with the glass the tips of the spore arms expand to form a pad or appressorium. Thus a high proportion of the spores have three appressoria, one at the tip of each arm in contact with the substratum. The fourth arm projects upwards into the water (see Fig. 7). Later one or more of the appressoria develop germ tubes. Tetraradiate spores caught in streams on cellophane-coated rods behave similarly. I believe that the high trapping efficiency of tetraradiate spores is due to the stability of the 3-point contact with the substratum.

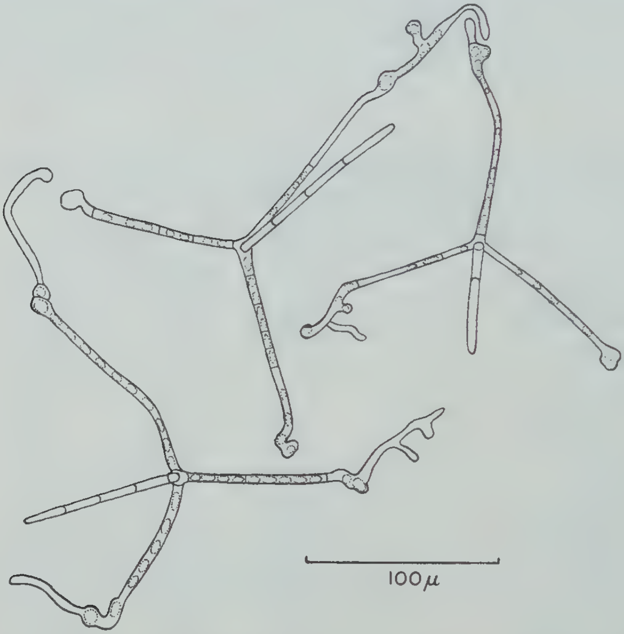


FIG. 7. Germinating spores of *Lemonniera aquatica*. The spores have settled to the bottom of a Petri-dish, and the three arm tips in contact with the glass have formed appressoria. The fourth arm projects upwards into the water.

The effectiveness of appressoria in attaching spores to surfaces can be tested experimentally. Spores are trapped on a cellophane or glass surface (collodion apparently inhibits spore germination) and counted. In one series of traps the spores are exposed immediately to the effect of wash-off in clean water. In a second series the traps are left in water for 12 hours for the spores to form appressoria, and then subjected to washing-off. Counts after the wash-off enable a calculation to be made of the per cent. spore loss for spores with and without appressoria. The results of such an experiment using *Tetrachaetum elegans* are shown in Table 4. Clearly the presence of appressoria improves adhesion to surfaces.

TABLE 4

The Effect of Presence of Appressoria on the Adhesion of Spores of Tetrachaetum elegans, *Attached to Cellophane-coated Traps and Subjected to a Water Flow of 31 cm./sec. for 1 hour*

	Without appressoria	With appressoria
Number of spores at first count . .	420	431
Number of spores at second count. .	121	353
Per cent. loss of spores . . .	73	19

In considering the results of these experiments it should be realized that they represent a very artificial system, using regular smooth surfaces and relatively high spore concentrations. There is evidence that roughening of the surface by the presence of particles of dirt on the trap, or by damaging the collodion film can result in an enormous increase in the deposition of spores around the rough area, leading to the formation of dense spore clumps impossible to count. Presumably the surfaces of leaves present rough surfaces for spore impaction, and it is clearly desirable to extend the experimental observations to rough surfaces.

Another problem raised by this work is the behaviour of sigmoid spores in rapidly flowing streams, and the details of their attachment. In the case of sigmoid ascospores (e.g. *Halophiobolus*) the presence of a sticky tip to the spore has been noticed by other workers, and it is assumed that these structures aid attachment. It is possible that similar structures occur in *Flagellospora curvula*, for when the spores of this fungus are allowed to settle in shallow dishes they frequently adhere at one end, with the body of the spore projecting upwards into the water. Attempts to demonstrate a differentiated structure at the tip of the spore have been unsuccessful, but it is possible that with suitable techniques such structures could be revealed.

SUMMARY

1. Evidence is presented for the evolution of tetraradiate propagules in a number of unrelated organisms as a probable adaptation to an aquatic environment, and speculations about the significance of such structures are discussed.

2. In direct observations of sedimentation rates no correlation could be found between spore shape and sedimentation rate. Tetraradiate spores did not appear to settle more rapidly than other spore types. The sedimentation rates of all the fungus spores studied were very low in relation to the current speeds in streams where the fungi are abundant.

3. In trapping experiments the trapping efficiency of tetraradiate spores was found to be considerably higher than that of other spore forms studied. These results were confirmed by trapping spores from mixed suspensions.

ACKNOWLEDGEMENTS

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Further Evidence for a Growth-Promoting and Flower-Inhibiting Hormone in Strawberry

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With two Figures in the Text

ABSTRACT

Further experiments with runner plants of the cultivated strawberry joined in pairs as donor/receptor units are reported. Stolon production was promoted in receptor plants in short days by long-day treatment of donor plants. This was a photoperiodic effect induced by low intensity light. Again, as previously, petiole length was increased and flower induction inhibited by these, or similar treatments. An attempt to demonstrate the translocation and activity of a vegetative-growth-inhibiting, flower-promoting hormone gave negative results.

It is argued that the photoperiodic control of the growth habit is mediated mainly by a transmissible vegetative-growth-promoting and flower-inhibiting hormone, which is produced in leaves and acts at the growing regions.

INTRODUCTION

THE seasonal cycle of growth of the cultivated strawberry plant in temperate regions is regulated principally by the day length and temperature. Long days favour the vegetative habit of growth, that is, long petioles, large leaves, and stolon production, whereas short days favour the induction of inflorescences, and bring about decreased vegetative growth. In previous papers (Guttridge, 1956 and 1959) it was suggested that the photoperiodic regulation of growth is mainly achieved by a regulating system and hormone that promotes the vegetative growth habit and inhibits flower induction. It was shown that the characteristic habit of growth in long day lengths was induced in daughter plants growing in short days, when parent plants attached to them by the stolon were subjected to long photoperiods or long light-break treatment. The response of daughters was increased when the parents were illuminated with full daylight 3 hours longer each day than their attached daughters. It is thought that the extra illumination promoted translocation of assimilates from parent to daughter, and that this resulted in increased transmission of the vegetative growth-promoting stimulus.

In the five early experiments significant promotion of stolon production in receptors was only once obtained and in this instance most of the increased stolon production was due to the promotion of emergence, as distinct from promotion of the early stage of initiation. Moreover, the possibility that the promotion was due to improved nutrition could not be excluded.

The first experiment now reported shows that stolon production can be

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induced in receptors by long-day treatment of donors and that this is a photoperiodic effect. Another attempt to demonstrate the activity of a vegetative growth-inhibiting hormone again gave negative results. The experimental data are discussed in terms of hormone regulation of growth.

MATERIALS AND METHODS

Redgauntlet, a variety of the cultivated strawberry (*Fragaria ananassa*, Duch.) was used in both experiments. Adjacent plants in a runner series were taken for donor/receptor units and rooted in separate pots as in previous work (Guttridge, 1959). During the experiments the plants were grown in J.I. No. 2 compost in 6 in. clay pots. For the first experiment the pairs were rooted in the glasshouse in May and June, and when established put outdoors until required for treatment on July 17. For the second experiment the plants were rooted in August into pots plunged in the runner bed in the field. These plants were lifted on September 6 and grown in the glasshouse until required for treatment on October 19.

The older member of each pair is designated 'parent' and the younger 'daughter'. Parent plants had 1–2 more leaves than daughter plants. Any pairs with damaged connecting stolons were rejected. Some plants developed tip-burn on the leaves, especially at the higher growing temperature 18·3° C. (65° F.). No remedy for this condition was found, and the cause is not known. The disorder was not confined to experimental plants. It reduced petiole length especially of the longer petioles, thus somewhat reducing the magnitude of treatment differences. In all other respects the plants grew well, being managed according to good horticultural practice.

The two-plant units were arranged for treatment in growth cabinets which had been constructed in pairs, back to back, so that the two separate member plants of a pair could be grown in different cabinets. The stolon joining them passed through a light-proof slit in the connecting wall between the two cabinets. The light intensity varied within a cabinet according to position from about 900 to 1,200 f.c. at leaf height. This intensity was obtained from ten 80 watt and four 40 watt Warm White fluorescent lamps supplemented by two 100 watt and one 75 watt incandescent lamps illuminating an area approximately 2 m. × 0·7 m. The lamps were screened from the plant area by two layers of window glass. The incandescent lamps were also used for 'low intensity' supplementary light for photoperiodic treatments. The temperature was regulated by a thermostat, which gave short-term temperature fluctuations normally of less than ±1·1° C. (2° F.).

Separate analyses of variance were calculated (*a*) for petioles of receptor plants of treated and control pairs (Figs. 1 and 2, Treatments A, C, G, and J) and (*b*) for control pairs in different photoperiods (Treatments E, F, L, M). Neither block differences themselves nor their interactions with other differences were significant in any of the analyses. In the analyses of receptor plants, standard errors were based on residual variances after elimination of differences between cabinets, differences due to different stolon connexions within cabinets and in the first experiment differences due to defoliation and its interaction with the previous differences.

EXPERIMENTAL WORK

First experiment. The four cabinets, arranged in two pairs (1 with 2 and 3 with 4), provided the experimental environments as shown at the top of Fig. 1.

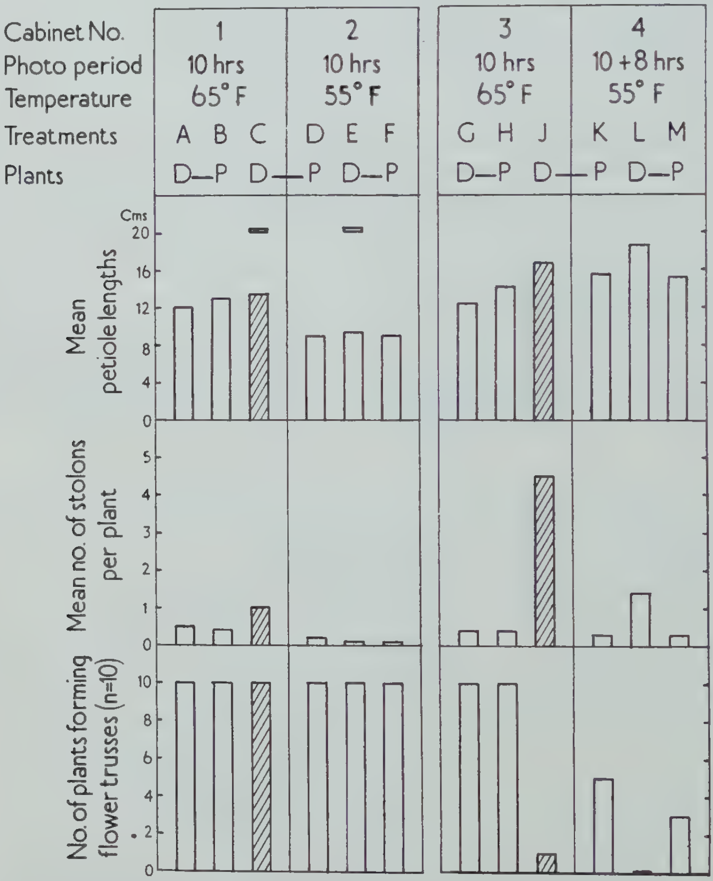

FIG. 1. Parent plants P and daughter plants D were connected as indicated. Petiole lengths are means of three successive petioles for plants growing at 18·3° C. (65° F.) and of two successive petioles at 12·8° C. (55° F.)

S.E. for comparing petiole lengths of treatments A, C, G, and J, is shown above the histogram for treatment C.

S.E. for comparing petiole lengths of treatments E, F, L, and M, is shown above the histogram for treatment E.

Histograms of the crucial treatments are hatched.

Ten hours daily illumination with the full lighting was given coincidentally in all cabinets. Supplementary illumination with the incandescent lamps increased the photoperiod in cabinet 4 from 10 to 18 hours. Cabinets 1 and 3 were operated at 18·3° C. (65° F.), cabinets 2 and 4 at 12·8° C. (55° F.). Cabinets 1 and 3 were therefore similar in all respects, while cabinets 2 and 4 differed from one another only in respect of the photoperiod.

The pairs of plants were arranged in the cabinets as shown in Fig. 1. The crucial treatments C and D, J and K were arranged with parent plants (P) in cabinets 2 and 4, that is at 12·8° C., and with daughter plants (D) in cabinets 1 and 3 at 18·3° C., with the stolons joining them passing through the light-proof slits in the connecting walls. The temperature difference was expected to provide favourable conditions for the translocation of assimilates from parent to daughter plants, by creating a highly active 'sink' in the daughter plants. Control pairs of plants were arranged entirely within each cabinet. There were ten pairs in each treatment, arranged in five blocks of two pairs. In one of the two pairs in each block the daughter plant was partially defoliated, so that in each treatment there were five pairs with both members intact and five pairs with daughter plants partially defoliated and parent plants intact. Old leaves were removed and the defoliated plants left with one fully expanded leaf and all younger developing leaves. This state was maintained by the removal of further leaves as the next younger leaves became fully expanded. Laminae only were removed, leaving the petiole intact for measuring at harvest. Outgrowing stolons and occasional branch crowns were removed at intervals to keep the plants to single crowns.

Treatments continued for 5 weeks, after which the pairs were separated and the plants grown for 3 weeks in long photoperiods in the glasshouse, during which time any flower buds initiated towards the end of treatment grew large enough to be easily recognized in dissection of the crowns at harvest.

Partial defoliation gave an overall reduction in petiole length due to the general lowering of vigour, but as the interaction with treatment was not significant, the results for defoliated and intact daughter plants have been pooled.

The direct effect of day length on plant pairs entirely within cabinets is represented by differences between E and L and between F and M (Fig. 1). Petioles were significantly longer in the long photoperiod than in the short. The long photoperiod gave slightly greater stolon production but decreased the number of plants initiating flower trusses (centre and lower histograms of Fig. 1). Comparison of treatments A with E and B with F shows that the petioles were longer at the higher temperature. Temperature, however, did not affect the number of plants with flower trusses and only slightly affected stolon production. This is not unexpected, as the short photoperiod probably pushed the threshold levels for these responses strongly towards the flower-inducing and stolon-inhibiting positions, out of range of the temperature effect. Darrow (1936) and Smeets (1955) have shown that temperature affects both these factors under other circumstances, and although no, or very small, measurable responses were obtained in the present experiment it is probable that the underlying tendencies were present.

The crucial comparison is between plants of treatment C, attached to parent plants D in a short photoperiod, and plants of treatment J, attached to parent plants K in a long photoperiod. This comparison is justified as the growth of the respective control daughter plants (treatments A and G) was almost identical in the two cabinets. The plants of treatment J had substantially longer petioles and more stolons than plants of treatment C, and, while all plants of treatment C initiated a flower truss only one plant of treatment J did so. Stolons of all stages of development up to emergence were promoted. The differences for stolon production and flowering are obvious and the full analysis of petiole lengths shows that the difference attributable to the crucial interaction cabinets × stolon-connexions is significant at 0·1 per cent. probability. Thus the photoperiodic stimulus was transmitted from parent along the stolon to daughter plants where it promoted the vegetative habit of growth and inhibited flowering.

Second experiment. The experiment was intended to see whether or not the transmission and activity of a vegetative-growth-inhibiting hormone could be detected. The treatments and results are tabulated in Fig. 2. Pairs of plants were arranged in the crucial treatments as in the first experiment, with parent plants at 12·8° C. and their daughter plants at 18·3° C. so that conditions were equally favourable for translocation of assimilates in the two experiments.

Daughter plants (treatment C) were arranged together with control pairs (A and B) in the cabinet in a long photoperiod of 10 hours' full light plus 5 hours' supplementary light at 18·3° C. The parent plants (D) attached to daughter plants (C), together with control pairs E and F were in cabinet 2 in 10 hours' full light at 12·8° C. Parallel conditions and arrangements pertained in cabinets 3 and 4, except that the photoperiod in cabinet 4 was extended to 15 hours by incandescent lighting, so that this cabinet differed only in photoperiod from its counterpart, cabinet 2. The 15-hour photoperiod was chosen for daughter receptors as being not too long to swamp any possible short-day stimulus carried along the stolon from donors in short photoperiods.

Although the two experiments were performed at different times and with differently raised plants the growth habit was similar under the same conditions of a 10-hour photoperiod at 12·8° C. (55° F.), as a comparison of the growth records for cabinet 2 in both experiments shows.

The supplementary illumination of cabinet 4 increased petiole length from about 8 cm. in short days to 16 cm. in long days (comparing treatments E with L and F with M). The 15-hour photoperiod at 12·8° C. failed to provide threshold stimulation for runner production and barely provided it for flower inhibition although the underlying tendencies were presumably present.

The growth of control plants was similar in cabinets 1 and 3; the slight differences in petiole length between treatments A and G are not significant. There is no suggestion in C of any inhibition of vegetative growth or promotion of flowering caused by the short photoperiodic treatment of parent plants D. Neither in fact is there any evidence for the promotion of vegetative growth in J caused by the long photoperiods on parent plants K. This latter negative response may be because the daughter plants, which were themselves in long photoperiods, were already sufficiently supplied with the hypothetical vegetative-growth-promoting hormone and additional supplies were useless.

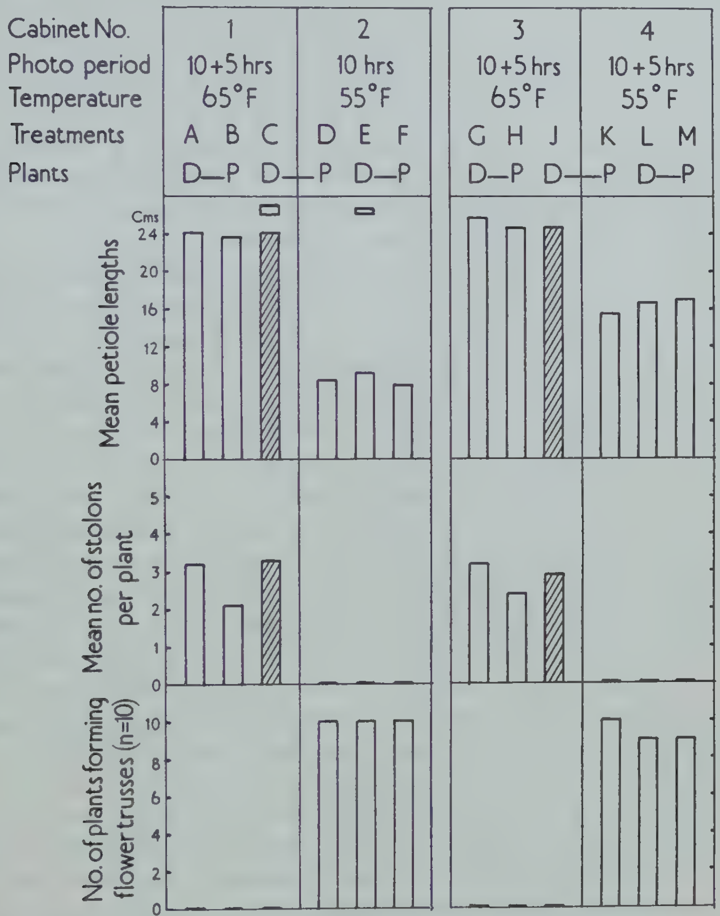

FIG. 2. Legend as Fig. 1.

DISCUSSION

The results of the first experiment demonstrate clearly the photoperiodic promotion of the vegetative habit of growth in daughter plants in short photoperiods by long-day treatment of parent plants attached to them by stolons. Petiole length and stolon production were increased and flower formation was inhibited. Stolons of all stages of development up to emergence were promoted.

It seems likely that all photoperiodic responses in strawberry are quantitatively regulated. This is demonstrated by the quantitative response of petiole length to photoperiod obtained by Borthwick and Parker (1952). It is not so obvious for stolon production and flower initiation, as they respond measurably only when threshold levels of stimulation or inhibition are attained. Quantitative differences and an apparently continuous response curve can, however, be obtained if many sites for response are provided, either by using large numbers of plants or by prolonged treatment, especially as thresholds may vary from plant to plant and with seasonal ageing or changing nutrition. The quantitative response of these factors is also indicated in another way by Hartmann's results (1947), which show that exposure of 50 per cent. of the total leaf area to long days, compared with all, or none, gave intermediate numbers of stolons and numbers of plants with flower trusses. Went (1957) has also shown that the suppression of flower induction is quantitatively related to the intensity of supplementary illumination.

There is extensive evidence suggesting that photoperiodically induced hormones are transported through the plant along with other assimilates chiefly in the phloem, and that, within limits, an increased flow of assimilate will be accompanied by increased transport of hormone, should it be in adequate supply. This hypothesis has been discussed generally by Lang (1952) and in relation to the present work in the previous paper (Guttridge, 1959).

Within the hormonal theory, and accepting this method of transportation, interpretation of the experimental facts can be based on any one of three postulates. Regulation of growth may be achieved by (1) a vegetative growth-promoting, flower-inhibiting hormone, (2) a flower-promoting, vegetative-growth-inhibiting hormone, or (3) both. The most obvious explanation of the results of both these and the previous experiments arises from adopting the first postulate, and suppose that a vegetative-growth-promoting, flower-inhibiting hormone, formed in parent plants in long photoperiods was transmitted along the stolon to the daughter plants where it directly promoted vegetative growth and inhibited flowering.

Adoption of the second postulate implies that vegetative growth is regulated quantitatively by a vegetative-growth inhibitor, whose production is high in short photoperiods, suppressing vegetative growth and promoting flower formation, and low in long photoperiods, permitting more vigorous vegetative growth and inhibiting flower formation. In the first experiment the increased vegetative growth of daughters in short days must then be expected to have resulted from dilution of the inhibitor by assimilates lacking or low in inhibitor, supplied by the donor plant in long days. The high temperature treatment is presumed to have created a highly demanding sink in daughter plants. Relative to the daughter plants at 18·3° C., however, the assimilation of parents at 12·8° C. would be low, hence, although appreciable translocation from parent to daughter took place, the transferred assimilates would be only a relatively small proportion of the total available to the growing regions of the daughter plants. It must be supposed, then, that the large effect on petiole lengths, stolon production and flowering, resulted from dilution of the vegetative growth inhibitor with small proportions of 'neutral' or 'low inhibitor' assimilates. This seems unlikely. Moreover, results of experiment 2 and of a treatment in an earlier experiment (Guttridge, 1959) do not support the suggestion of regulation by a vegetative-growth inhibitor. In these experiments parent plants in short days did not inhibit the vegetative growth of daughter plants in long days, although conditions appeared to favour translocation of assimilates from parent to daughter, as in the first experiment. If the experimental facts are to be explained by assuming a vegetative-growth-inhibiting hormone, then it appears that this hormone had no effect when introduced in relatively small amounts into plants low or lacking in inhibitor (second experiment) but a relatively small dilution of the inhibitor in plants already well supplied, effected a 24 per cent. increase in petiole length in the first experiment and a 104 per cent. increase in the earlier experiment (Guttridge, 1959). The dosage/response relationship implied here would not be expected for a hormone regulating petiole length, with its continuous response curve.

The absence of an inhibitory effect in daughters in the second experiment could also be explained by supposing the destruction in long photoperiods of the vegetative-growth inhibitor brought along the stolon from the parent plants in short photoperiods. To be destroyed in any but very small quantities by light the inhibitor would have to enter the leaves of the receptor plant. It is unlikely that the inhibitor would be transported into mature leaves for there is no reason to suppose assimilates are transported into them. On the other hand assimilates and inhibitor would certainly be transported into immature leaves, and its destruction in them would account for the absence of its effect on petiole length. In either case it can hardly account for the absence of runner inhibition (or the absence of flower promotion in earlier experiments where thresholds were attained), unless it is supposed that assimilates pass through intermediate leaves on the way to the terminal and axillary growing points in the bud. This is unlikely in the strawberry as the path of assimilates from base to top of the crown would be increased several-fold and apparently unnecessarily by the detour. The hypothesis that long photoperiods destroy the vegetative-growth inhibitor involves this awkward corollary.

The first hypothesis, involving a vegetative-growth promoter, which also inhibits flower induction, is more acceptable as it does not involve unlikely dosage/response relationships or unnecessarily devious paths of translocation. This hypothesis also accords well with the fact that post-harvest defoliation, as a commercial practice, increases the crop the following year (Wilson and Rogers, 1954) by increasing flower initiation (unpublished data) supposedly by removing the source of flower inhibitor.

The third alternative, namely that both promoters and inhibitors are operating in the strawberry cannot be excluded, as any argument for either the first or second explanation cannot exclude the possibility that both regulators were present but with one dominant over the other. Indeed the possibility of a multiplicity of hormones cannot be excluded. However, it does not appear necessary to postulate the existence of more than one in the strawberry. This is because, allowing for the need to reach thresholds in order to obtain measurable effects on flower initiation and stolon production, these responses and petiole length are all affected concomitently by change in photoperiod. In the current experiments, supplementary illumination always increased petiole length. Whenever changes were induced in stolon production or flower formation, the extra lighting increased the number of stolons and reduced flower formation. These parallel responses have been observed in other experiments and in published work (Darrow and Waldo, 1934; Hartmann, 1947). To accept that photoperiodic regulation of both vegetative growth and flowering is achieved through a single hormone does not imply any constant relationship between flower initiation, stolon production, and petiole length, for each of these can be expected to be differently affected by nutrition, temperature, light intensity, and other environmental factors. Went (1957) found flower induction was associated with a range of petiole lengths.

It is thought therefore that these results are good evidence for supposing that the photoperiodic regulation of growth in the strawberry is mediated chiefly by a hormone which is produced in long days or formed in greater quantity in long days. This hormone, when transferred to the responsive sites, promotes vegetative growth and inhibits flowering.

It is interesting that gibberellic acid acts as a substitute for long photoperiods (Brian, 1958; Brian *et al.*, 1959). Gibberellin A_1 (MacMillan and Suter, 1958) and other gibberellin-like factors (Phinney and Neely, 1958; West and Murashige, 1958) have been extracted and identified from plant tissue. Thus hormones exist in plants which have some properties similar to the postulated vegetative-growth promoter. Gibberellic acid has in fact been found to induce responses in the strawberry characteristic, in part, of the long-day-growth habit (Guttridge and Thompson, 1959; Thompson and Guttridge, 1959).

Lang (1952) has discussed in detail flower-inhibitory effects in other plant species, arguing that the evidence then available for the existence of a *transmissible* flower-inhibiting substance was very poor. He pointed out that the observed inhibitory effects 'seem to be directed, not against their functioning, but against the formation of flower-promoting substances'. In the strawberry experiments it appears that the 'inhibitory effect' was transmitted along the stolon, and for it to act against the formation rather than against the functioning of a floral stimulus, it would have to be detoured into the leaves of the receptor plants. Such a detour is unlikely as was argued earlier in the discussion.

Lang also points out that for a non-induced leaf to be inhibitory it must be positioned between the induced leaves and the recepting sites (Harder, 1948), and may therefore exert its inhibitory effect by blocking transportation of the flowering stimulus. In the strawberry experiments the 'non-induced' leaves (i.e. those in long days) were not so located but exerted their flower-inhibitory effects from the remote and morphologically lower parent plant. If such a position is, in fact, favourable for supplying the growing regions of a daughter plant and a flower-promoting stimulus is involved, then it is pertinent to inquire why in the second experiment the 'favourably positioned' parent in short days did not induce flowering and associated vegetative effects in the daughter plants.

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Fixation of Nitrogen by Non-nodulated Seed Plants

BY

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Abstract

Evidence has been collected from published records of the natural development of soils and vegetation, and also of some cultivated soils under crop plants, to show that many cases exist where there is an unexplained increase in soil nitrogen of the order of 50 lb./acre/year.

Pot experiments with a number of pioneer plants which were grown for 6 months to 3 years in a minus-nitrogen sand culture showed considerable gains in nitrogen in the absence of the classical non-symbiotic nitrogen-fixing micro-organisms, *Azotobacter* and *Clostridium*. Seedlings of *Pinus radiata* with mycorrhiza formed from different fungal cultures grew successfully in the no-nitrogen culture when non-mycorrhizal plants died.

When some plants growing in the no-nitrogen culture were exposed to an atmosphere enriched in ^{15}N, an uptake of molecular nitrogen occurred with the growth of the following non-nodulated plants: *Coprosma robusta* and *Prunus armeniaca* absorption in leafy shoots; *Pinus radiata*, *Dactylis glomerata*, and *Epilobium erectum* absorption by whole plants and by roots exposed separately.

Introduction

It is widely recognized that there are still basic problems presented by the balance between soil and plant nitrogen, and the sources of supply by which this nitrogen is built up. It has been pointed out by Allison (1955) that soil nitrogen balance sheets do not tally. When Lawes and Gilbert at Rothamsted began their studies which led them finally to the conclusion that green plants could not use the nitrogen of the air, they found from field experiments not only that legumes gained nitrogen but also that unmanured turnip crops showed large, unexplained increases in this element. In a manurial trial in Canterbury, New Zealand, on a crop of rape growing in a poor soil, Walker *et al.* (1954) found that the application of sulphur and phosphorus alone increased the yield of nitrogen in the crop nearly forty-fold. The famous unmanured wheat plot on Broadbalk field continues to give a steady yield at a low level (Russell and Russell, 1950) and it has been pointed out by the same authors that some semi-arid wheatlands in Australia and America have carried a large number of wheat crops without soil nitrogen being depleted and without the yield showing any noticeable decrease, under conditions when no nitrogen has been added as fertilizer to balance what was taken away in the grain. Forster (1950) for Victoria and Drover (1956) for Western Australia have given figures for experimental plots maintained under continuous wheat without loss of soil nitrogen, and Lyon and Bizzell (1934) gave similar figures for plots in America under continuous cereals. Plots kept under

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continuous ryegrass in Western Australia have been found by Parker (1957) to show an annual increment to the soil of 50 lb. N/acre. Wilsden and Ali (1922) who made calculations of nitrogen removed in the crop and remaining in the soil in cultivated land in the Punjab mainly under wheat, showed that over a long period there had been an unexplained gain of 38 lb. N/ac./yr.

A number of records of long-term trials with tree crops have also revealed anomalies with regard to nitrogen. Dullum and Dalbro (1956) tell of an apple trial over 24 years in which trees with no added nitrogen showed no reduction in tree size or yield. Oland (1955) also reported no yield response to nitrogen fertilizers on apple crops. For tung trees it was found by Neff *et al.* (1953) that the amount of nitrogen removed in the crop was far greater than the amount applied as fertilizer, yet the quantity of nitrogen in the leaves rose and the trees increased considerably in size. In field trials with orange trees in southern California Wallace *et al.* (1952) found no yield response to nitrogen fertilizer and after 9 years there were no detectable symptoms of nitrogen deficiency on trees which had received no nitrogen. Similar results are reported from Australia by Frith (1952) and Bouma (1956) who report continuance of healthy tree growth and sustained yield in a series of orange trees which received no nitrogen fertilizer. Pratt, Goulben, and Harding (1957) found that in an irrigated citrus orchard in California the soil of unfertilized plots showed no change after 27 years.

When we turn to uncultivated land the problem of where the plants obtain their supplies of nitrogen is more acute. Original mineral soil or depleted land may contain little or no nitrogen, yet, after the growth of many kinds of non-leguminous plants in such a medium, a large amount of organic matter containing nitrogen is built up. A study of Blackland soil in Texas by Smith *et al.* (1954) showed that where an eroded plot was left for 20 years under native vegetation of grass, there resulted a gain of 30 lb. N/ac./yr. At Rothamsted a plot on Geescroft field which was allowed to go derelict mainly under the grass *Aira caespitosa* was found to gain nitrogen just as fast as other plots mainly under legumes (Russell and Russell, 1950). In New Zealand the rapid growth of exotic pines on depleted mountain country and even on sand dunes is striking. The writer measured the nitrogen in a 25-year-old *Pinus radiata* forest on depleted hill country near Nelson, and found the gain in nitrogen per acre to be approximately 300 lb. in the standing trees and 500 lb. in the litter, and average increment of 32 lb. N/ac./yr. Dickson and Croker (1953) in a detailed study of plant sequence on a mineral soil found that under a forest, predominantly *Pinus ponderosa*, there was a relatively rapid build up of soil nitrogen in the first 60 years with a gain of 56 lb./ac./yr. From the flourishing character of the pines colonizing the mineral soil these authors suggest that the mycorrhiza merits examination for possible nitrogen fixation. Development of soil under *Pinus* plantations on dune lands in Scotland has been investigated by Ovington (1951) who has found a small build up in soil nitrogen under the conifers in addition to what was gained by the trees. A later study by Ovington (1956) in the development of woodland conditions and changes in soil under plantations of both coniferous and dicotylous trees at different locations in England has shown considerable gains in nitrogen in standing trees, their litter, and the mineral soil beneath them, over and above the amount present on comparable unplanted plots.

Some non-leguminous weeds grow normally under conditions of very low nitrogen, e.g. *Atriplex hastata* (Rees and Sidrak, 1955 and 1956) and Amaranthus spp. (Woo, 1919, and Clements, 1920), yet they may contain exceptionally large amounts of nitrogen. Some of these such as *Chenopodium album*, &c. (private communication M. R. Coup, Ruakura Animal Res. Sta., Hamilton) and *Silybium marianum* (Glue, 1957) occasionally cause nitrite poisoning in ruminants due to their excess content of nitrate, while *Amaranthus*, quoted above, has been recommended as a fodder plant because of its high content of nitrogen. Many other non-leguminous herbs are conspicuously successful in places where soil nitrogen is negligible. Studies of the plant colonization of pit heaps by Brierley (1956) and Hall (1957) give lists of species, mainly non-leguminous, which become established in the rock debris. After 10–50 years of plant growth on these heaps an organic soil accumulates.

Thus it appears that there are many cases of a substantial gain in soil nitrogen of 30–50 lb./ac./yr. in the absence of legumes or fertilizers. The only known factors which supply nitrogen to the soil are firstly, rain, and secondly, non-symbiotic nitrogen-fixing micro-organisms. Nitrogen added by rain varies from place to place; figures from 4–10 lb. N/ac./yr. are given by Goldschmidt (1954). Drover and Barrett-Lenard (1956) made careful measurements at a number of stations in Western Australia and found the total nitrogen in the rain to be from 0·6–3·7 lb./ac./yr. Analyses made by the writer of samples of rain at Nelson gave a figure of about 1 lb./ac./yr. The numbers and activity of the non-symbiotic micro-organisms have been examined by several workers, Jensen and Swaby (1940), Parker (1954) and (1955), Beadle and Tchan (1955), and Tchan and Beadle (1955), whose general conclusion was that the effect of these organisms was insignificant. Careful measurements made by Delwiche and Wijler (1956) using isotopic nitrogen have shown that fixation under conditions thought to be favourable and with a heavy inoculation of *Azotobacter* amounted at the most to only one or two pounds per acre. However, as these non-symbiotic nitrogen-fixing bacteria are found abundantly only in fertile, cultivated soils and are absent from forests and low-fertility areas they cannot be an important factor in maintaining the balance of soil nitrogen.

A claim by Ingham (1950) that substantial amounts of ammonia could be absorbed directly from the atmosphere has not been confirmed by the writer who repeated his experiments in Nelson. The gains claimed by Ingham may have been due to his use of acid-washed filter-papers which always contain measurable amounts of nitrogen.

Against the addition of nitrogen from these causes we have to weigh the loss in drainage water and the loss by denitrification. That these are considerable is indicated by the work of Lipman (1936), Pinck and Allison (1947), MacVicar *et al.* (1950), Jones (1951), Broadbent and Stojanovic (1952), Wijler and Delwiche (1954), Hauck and Melsted (1956), Volk (1956) and others. Goldschmidt (1954) considered that all the nitrogen brought down in the rain reached the ocean. The losses in drainage water and by denitrification may, in fact, more than offset the gains due to rain and non-symbiotic fixation.

Thus when a balance is made of all the losses and gains which have been measured, both for some agricultural soils and for other soils under natural conditions of plant growth, we find that there is in many cases an annual gain in nitrogen of up to 50 lb./ac. The only explanation for this which seems probable at present, is that the growing plants in these soils have utilized atmospheric nitrogen, either by means of some symbiosis with micro-organisms or by some activity of their own tissues.

There have been a number of earlier experiments designed to throw light on this problem. Over a long period Lawes and Gilbert (1861) and (1889) worked at Rothamsted to discover whether or not green plants could use nitrogen from the air. They grew plants under rigid conditions of nitrogen starvation and finally concluded, in accordance with the work of Hellriegel and Willfarth, that no other green plants than legumes could use atmospheric nitrogen. However, they also recorded the results of M. Ville and later of M. Joulie who, from apparently well-conducted pot experiments, concluded that some plants other than legumes could use nitrogen from the air. Ludwig (1934), who investigated the growth of willow cuttings in nitrogen-free water culture, found, after 2 months' growth, small but statistically significant gains in nitrogen; but as growth ceased at this point the work was not considered to be conclusive. As trace elements were not added to the culture solutions used in these experiments the lack of them may have affected the results. Other claims to have demonstrated the use of atmospheric nitrogen by non-leguminous plants (Lipman and Taylor, 1924; Brown, 1933; and Ruben *et al.*, 1940), were clearly unsatisfactory (Burris, 1941). This earlier work presents a number of contradictions; but by the older methods it was difficult to obtain a clear result. The stable isotope of nitrogen, however, is a new tool well suited to the problem of testing a plant's ability to use the gaseous element, and thus the experimental work described in the following section of this paper has included the use of ^{15}N.

EXPERIMENTAL

General Pot Experiments

A selection of plants which grow normally in New Zealand under poor soil conditions was grown in sand culture in polythene buckets in a strictly no-nitrogen medium; for comparison *Lolium perenne*, wheat and uninoculated clover which normally require nitrogen were also included in the series. A fairly coarse nitrogen-free quartz sand was used with a culture solution of the following composition; Mg 3, Ca 8, K 3, Na 1, SO_4 8, PO_4 15, CI 2, Fe 0·1, Mn 0·02, Cu 0·002, Zn 0·002, Mo 0·005, BO_3 0·1 mg. equivalents per litre, adjusted to pH 5 with H_2SO_4. As it was difficult to keep the Ca in solution some $CaSO_4$ was added dry to the pots. Cultures were started with unsterilized seed or with carefully washed seedlings which had been raised in a natural soil and which were presumed to carry a normal complement of micro-organisms. The acid medium free from organic matter was designed to prevent the growth of the classical non-symbiotic nitrogen-fixing micro-organisms, *Azotobacter* and *Clostridium*, but at different times the medium was tested for their presence by methods recommended to the writer by Dr. Jane Meikleljohn of Rothamsted. As all tests were negative it was assumed that these organisms played no part. The surface of all pots was shaded with a layer of pebbles to prevent the growth of algae.

In the strictly no-nitrogen medium *Pinus radiata* continued to grow for 3 years steadily though slowly, but, for the other woody plants tried in this series, *Coprosma robusta*, *Leptospermum scoparium*, *Aristotelia serrata*, and *Nothofagus fusca*, growth was fairly vigorous for the first 6 months, slowed down during the following 6–9 months, and practically ceased beyond about 18 months. *Dactylis glomerata*, *Raphanus sativus*, and *Epilobium erectum*, which were grown for shorter periods, showed typical signs of starvation but, nevertheless, they made a fair amount of growth. In *D. glomerata* the outer leaves and leaf tips yellowed, then died, and in the herbs *R. sativus* and *E. erectum* plants were small with strong red coloration. *Lolium perenne* and wheat germinated well, then remained alive, making almost no further growth; uninoculated clover (surface-sterilized seed) germinated well but died after 3–4 weeks. Dried material from these pot experiments was analysed to determine the gain in nitrogen with the results shown in Table 1.

Where growth was sustained in the strictly no-nitrogen culture the plants showed considerable gains in nitrogen which were greatest for *P. radiata*. Plants of *L. perenne* which remained practically stationary showed an insignificant gain.

TABLE 1

Nitrogen Contents of Experimental Plants

Plant	Period of growth months	mg. N/ seedling	mg. N/ plant reaped
Pinus radiata	14	1·0	13·4
,,	25	1·0	32·3
Coprosma robusta	14	0·09	3·7
Leptospermum scoparium	12	0·065	3·4
Nothofagus fusca	9	0·24	2·8
Dactylis glomerata	6	0·014 (seed)	0·26
Epilobium erectum	6	0·010	0·90
Raphanus sativus	4	0·24 (seed)	1·76
Lolium perenne	6	0·03 (seed)	0·05

In order to test the possibility that these pioneer plants were able to use traces of ammonia which might be present in the atmosphere, one set of the pots of *P. radiata*, *C. robusta*, *N. fusca*, *Leptospermum scoparium*, *A. serrata*, and *E. erectum* was kept for 4 months in a cabinet through which scrubbed air free from any trace of ammonia was pumped. These plants grew equally as well or slightly better than those on the open bench.

Pinus radiata *Pot Experiment with Different Mycorrhizal Fungi*

As *P. radiata* plants with natural mycorrhiza had grown well in the minus-N sand culture an experiment was set up to compare non-mycorrhizal plants with those which had mycorrhiza. Seed of *P. radiata* was surface sterilized by washing in 0·1 per cent. $HgCl_2$ and sown in sterile sand watered with sterile tap water. Four months later the seedlings which had well-developed roots were planted aseptically in sterilized perlite in clay pots to which the following additions were made:

1. Culture of *Amanita muscaria* (Swedish isolate).
2. Culture of *Boletus luteus.*
3. Culture of *Rhizopogon rubescens.*
4. All three cultures mixed.
5. Sieved air-dry *Pinus* soil from Taita.
6. Same soil autoclaved 3 weeks previously and subsequently well aerated.

Mr. John Gilmour of the Forest Research Institute, Rotorua, kindly prepared the fungal cultures for this experiment. Approximately 100 ml. volume of each of the six materials was mixed with the perlite in six-inch clay pots in each of which 10 seedlings were planted. The pots were placed in large plastic bags and kept in the glasshouse for 3 months. At this stage the plants were of even size with shoots about 12 cm. high; lots 3, 4, and 5 were slightly darker green than the others. When some from each lot were lifted and the roots examined their appearance was as follows:

1. No mycorrhiza though some fungal hyphae were seen on the long trailing roots.

2.
3. All showed short, brown, lateral, dichotomous mycorrhizas which were most numerous in lots 3 and 4.
4.
5.
6. No mycorrhizas and no fungi on the root surfaces.

From each lot, 15 seedlings were planted out in lots of 5 per bucket in polythene buckets which had been washed out with formalin and filled with fresh perlite, a practically sterile material. These were kept in a closed glasshouse and watered with minus-N culture solution. Results are summarized in Table 2.

A set of sterile seedlings raised later was grown in sterile culture with the addition of 1 mg. eq. N as NH_4NO_3 to the basic culture solution. These non-mycorrhizal plants which were kept for 6 months sustained a rapid healthy growth in striking contrast to the non-mycorrhizal plants in minus-N culture which quickly died. The mycorrhizal plants on the other hand survived and continued to grow slowly when deprived of nitrogen, some fungi apparently stimulating growth more than others.

TABLE 2

Growth Responses of Treated Plants (p. 627) *in the Absence of Combined Nitrogen*

Lot	After 1 month	After 2 months	After 5 months
1	Green and healthy	Beginning to die at tips	7 plants dead, 8 nearly dead
2	Green and healthy	Dark-green, growing	Increased shoot length
3	Dark-green, growing	Dark-green, growing	Increased shoot length
4	Dark-green, growing	Dark-green, growing	Increased shoot length
5	Green and healthy	Paler green and less growth than 2, 3, and 4	Same colour as 2, 3, and 4 but less growth
6	Beginning to die at tips	All plants dead	

^{15}N *Experiments*

In collaboration with Mr. T. A. Rafter of the Nuclear Science Division of the New Zealand Department of Scientific and Industrial Research, a number of experiments using isotopic nitrogen have been carried out. Enriched nitrogen gas was prepared from labelled ammonium sulphate and sodium hypobromite in a vacuum apparatus designed and built by Mr. Rafter. The gas was bubbled through dilute and concentrated sulphuric acid and passed through a double liquid air trap to ensure that no traces of ammonia or oxides of nitrogen were present. Plants in the no-nitrogen culture were grown in specially made glass pots, with lids fitting on ground-glass flanges, into which the enriched atmosphere could be introduced. In the first series the pressure in the pot was first reduced to half atmospheric then restored with cylinder oxygen; then reduced a second time and restored with enriched nitrogen. At the end of an experiment most of the atmosphere was recovered by pumping it into the vacuum apparatus. It was then used to fill another pot in which the pressure was first reduced to half. This procedure diluted the atmosphere by half its enrichment each time it was used. A different method was followed in the latter experiments. The pot containing growing plants was first flooded with culture solution and the prepared or recovered atmosphere was introduced by water displacement.

The plant material was reaped immediately, cut into small pieces of about 1 mm. length and dried at 80–90° C. The combined nitrogen was extracted and recovered by semi-micro Kjeldahl procedure, roots and tops of plants as far as possible being treated separately. Many controls were carried out by processing comparable plants which had not been exposed to an enriched atmosphere, or, in two cases mentioned, autoclaved plant material which had been included in the ^{15}N experiments was treated as a control. Results are presented in Table 3. These figures show that for a number of plants a significant amount of molecular nitrogen has been transformed into plant protein in the space of a number of days.

In Expt. 1, plants of *C. robusta*, *P. radiata*, and *Lolium perenne*, growing in the no-nitrogen sand culture, were exposed to an enriched atmosphere in the same pot. After 12 days the *C. robusta* and *L. perenne* were enriched but not

TABLE 3

Enrichment of Sample Tissues from Atmospheres Containing ^{15}N

Period of Experiment		Atmosphere Atom % excess				Atom % excess ^{15}N	
days		^{15}N	O$_2$%	Plant		Enrichment	Control
1	12	15	12	*Coprosma robusta*	tops	0·190	0·006
					roots	0·012	0·004
				Pinus radiata		0·006	0·004
				Lolium perenne	tops	0·080	0·006
					roots	0·222	0·006
2	14	8	29	*L. perenne*	tops	0·003	0·006
					roots	*	0·006
3	25	8	17	*Dactylis glomerata*	tops	0·16	0·004
					roots	0·15	0·005
				Raphanus sativus		0·010	0·006
				Nothofagus fusca		0·017	0·003
				Prunus armeniaca (shoots)		0·009	0·005
4	14	5	27	*Dactylis glomerata*	tops	0·05	0·004
					roots	0·052	0·003
5	30	0·8	20	*D. glomerata*	tops	0·006	0·005
					roots	0·022	0·005
6	21	3	24	*D. glomerata*	tops	0·06	0·005
					roots	*	—
7	18	16	20	*D. glomerata*	tops	0·016	—
					roots	0·225	—
8	10	*c.*10	10	*D. glomerata*	tops	0·003	—
				(roots only exposed)	roots	0·014	—
9	10	*c.*10	10	*D. glomerata*	tops	0·008	—
				(tops only exposed)	roots	0·005	—
10	8	60	10	*D. glomerata*	tops	0·045	—
				(roots only exposed)	roots	0·22	—
11	18	11	29	*C. robusta*	tops	0·041	0·005
					roots	0·105	—
12	7	31	24	*P. armeniaca* (shoots)		0·058	0·005
				C. robusta (shoots+glands)		0·024	0·005
				,, (shoots+glands)		0·047	(autoclaved)
13	14	7	27	*P. radiata*	tops	0·056	0·005
					roots	0·029	—
14	9	*c.*15	20	*P. radiata*	tops	0·004	—
				(roots only exposed)	roots	0·042	—
15	14	25	15	*Epilobium erectum*	tops	0·51	0·005
					roots	0·104	(autoclaved)
16	28	6	40	*E. erectum*	tops	0·07	0·006
					roots	0·07	0·005
17	18	6	20	*E. erectum*	tops	0·020	—
					roots	0·095	—
18	10	60	10	*E. erectum*	tops	0·003	—
				(tops only exposed)	roots	0·003	—
19	68	60	10	*E. erectum*	tops	0·003	—
				(roots only exposed)	roots	0·33	—

* Sample lost

the *Pinus*. As the result for the ryegrass was in disagreement with the pot experiments which showed that this grass in the no-nitrogen culture made no progress, it was thought possible that root transference had taken place. In Expt. 2, where *L. perenne* alone was exposed to an enriched atmosphere, no enrichment was found in the plant material. The behaviour of ryegrass in further pot experiments was anomalous. On the whole the plant remained alive but made little or no growth in the no-nitrogen culture, except that in a few cases where single plants of *L. perenne* were potted with *Pinus* or *Coprosma* the grass grew fairly well. In an attempt to determine if nitrogen passed to the grass by root transference, enriched seedlings of *C. robusta* and *P. radiata* were saved from subsequent ^{15}N experiments and planted in small pots with ryegrass in no-nitrogen culture. After 14 days when the first grass plants together with *C. robusta* were reaped, dried, and extracted, the enrichment was still in the *C. robusta* and nothing had been gained from it by the ryegrass. The *P. radiata* pot was left for a much longer period, 110 days, but still no enrichment passed to the ryegrass. Thus it is still not clear how the ryegrass plant in the first experiment gained so much nitrogen in 12 days.

In Expt. 3 a further group of plants, *Dactylis glomerata*, *Raphanus sativus* and *Nothofagus fusca*, growing in the same pot, together with cut spring shoots of *Prunus armeniaca* (cultivated apricot) standing in a small beaker of culture solution, were exposed to an enriched atmosphere. A significant enrichment occurred for each plant. In all seven further experiments with cocksfoot grass alone, have been carried out and in each case there was a significant enrichment in the plant material except in Expt. 9 where only the tops of the plant were exposed. Under the conditions of these experiments in the strictly no-nitrogen culture, the growing grass utilized atmospheric nitrogen apparently through its roots. This result is in accordance with both the pot experiments and field evidence.

A further experiment (11) with *C. robusta* again showed a significant enrichment which, however, was mainly in the roots, whereas in Expt. 3 enrichment was mainly in the shoot. For this particular experiment the distribution of the enrichment may have been affected by a delay of 3 days which occurred between opening the pot to the air and reaping the plant material. Two other experiments in which roots alone of *C. robusta* were exposed gave inconclusive results. In the next experiment cut shoots, 8–10 cm. long, of *C. robusta* and of apricot, standing in small separate beakers of nitrogen-free culture solution, were exposed to an enriched atmosphere. A tube of autoclaved shoot material was included for a control. From one set of *C. robusta* shoots the stipular glands were removed. In each case a significant enrichment was measured; removal of the stipular glands appeared to favour rather than hinder the process. This experiment showed that fixation could take place in the leafy shoots, and the absence of any enrichment in the autoclaved material showed that no exchange mechanism operated.

P. radiata was further tested both by exposing whole plants (Expt. 13) and roots alone (Expt. 14) with positive results. Fixation definitely occurred in the roots. Another experiment in which a shoot alone was exposed gave a negative result.

The weed *Epilobium erectum*, which was tested in five separate experiments, showed in all cases where roots were exposed a significant gain in the isotope, which indicated that nitrogen fixation had taken place with the growth of this plant. A tube of autoclaved shoots exposed in Expt. 15 remained without enrichment, showing that fixation depended on growth of the living plant.

For this plant also Expts. 18 and 19 were conducted exposing the shoots only, or the roots only. The response of *E. erectum* was the same as that of cocksfoot grass and showed no absorption by the leaves but a positive fixation by the roots. Both *E. erectum* and *D. glomerata* were tested for nitrogen fixation when they were growing in a poor soil/sand mixture with a total nitrogen content of 0·03 per cent. The experiments were run for 14 days after which period plant and soil nitrogen were recovered separately, but no fixation was found in either plant under these conditions and none was detected in the soil. Although total nitrogen in the potting mixture was low, the available mineral nitrogen may have been so improved by mixing and aerating the soil and sand in the potting process that sufficient was present for the plants for the duration of the experiment.

An effort was made to raise some of the plants in sterile culture. *P. radiata* which is normally mycorrhizal would grow in the no-nitrogen culture only when the mycorrhiza was well developed. *C. robusta* normally has poorly developed endophytic mycorrhiza present only in part of the root system, but when surface-sterilized seed was sown in tubes of sand plus no-nitrogen culture solution, or of soil/sand mixture, growth of the plants was extremely slow. Surface-sterilized seed of *D. glomerata* germinated readily and apparently sterile seedlings were grown in 750 ml. conical flasks on sand plus culture solution. An assay of the nitrogen in one series of cocksfoot grown in this way without nitrogen, and of another series grown with a small addition of nitrogen, showed that no significant gain had resulted in either case after 5 months' growth.

DISCUSSION

The results recorded in this paper show that under some conditions at least a number of non-nodulated plants are able to utilize atmospheric nitrogen on a scale which, though less than that of nodulated plants, is still sufficient to make an important contribution to their nutrition. In some cases uptake of nitrogen gas occurs in leafy shoots, in other cases, e.g. in *P. radiata*, *D. glomerata*, and *E. erectum* it takes place in the roots. A single negative result such as that reported by Bond and Scott (1955) for *P. sylvestris* mycorrhiza cannot be taken as a final statement. The different plants used in the experiments reported here have shown considerable variation in their responses. Further, in order to obtain a clear result from an experiment it is necessary that the plant should be well established in a growing medium, either without available nitrogen or with an insufficient supply, so that it will continue to grow and carry out any fixation process it may be capable of while it is exposed to isotopic nitrogen.

It is possible that some similar nitrogen fixation occurred with the growth of the grasses *Agrostis tenuis* and *Festuca rubra* in the detailed work reported by Blackman and Templeman (1940). These authors found that when pots were shaded, plants receiving no nitrogenous fertilizers actually contained more total nitrogen than those which were supplied with nitrogenous fertilizers, although under conditions of full daylight, nitrogen compounds supplied through the soil greatly stimulated growth and yield.

The evidence given by isotopic nitrogen in the experiments recorded in this paper is clearly in agreement with a great deal of field evidence. It suggests that nitrogen fixation is a commoner process in the growth of green plants than has been assumed, and that it may take place in leafy shoots as well as in roots. From what is already known of the effect of symbiotic associations and of the high degree of specialization of the legume nodule, it would appear possible that less effective, loose associations could occur between micro-organisms and the roots of other plants, such as cocksfoot grass and *Epilobium* spp. On the other hand, for a micro-organism to make a symbiosis with a higher plant sufficiently effective to produce the amounts of fixation shown by the results of this research, one might expect it to be moderately conspicuous. Careful examination of cocksfoot roots from the no-nitrogen cultures showed bacteria to be present occasionally on the root surfaces, but not in great amount. Endophytic mycorrhiza, on the other hand, was regularly seen and this may have caused the nitrogen fixation. It will be necessary to test separately sterile plants, those with mycorrhiza, and with bacterial associates before the effective mechanism can be distinguished. In order to determine finally the importance of micro-organisms associated with higher plants more work will be necessary with sterile cultures and with controlled combinations of higher plants and isolated micro-organisms.

Nitrogen fixation which has been detected in leafy shoots such as the glandular, red-tinted, apricot shoots, or in the yellow-green *C. robusta* shoots, which have red glandular hairs in domatia and dark-red stipular glands, likewise could either be due to associated micro-organisms, or to some function of the higher plant tissue. Some efforts have been made to isolate an effective organism from these leaf glands but so far efforts have not been successful. The red-tinted spring shoots of apricot are similar to the shoots of many other trees which thrive on poor soil and which may be found to have a similar capacity for nitrogen fixation. The problems associated with the growth and nutrition of various pioneer plants seem to be worthy of further investigation.

SUMMARY

1. A review of the relevant literature has shown that for many kinds of cultivated land there is an inexplicable gap between the amount of nitrogen added to the soil and the amount removed in the crop.

2. For native vegetation pioneering poor sites there is an acute problem as to where the nitrogen comes from which is found in the organic soil built up within a period of years of growth of many kinds of non-nodulated plants.

3. A balance sheet of the known gains and losses of nitrogen in such soils shows that many cases exist where there is an unexplained increment of the order of 50 lb.N/ac./yr.

4. Some selected pioneer plants have been grown in minus-N sand culture and although growth was slow some made considerable gains in N.

5. A number of experiments have been carried out in which plants growing in minus-N culture have been exposed to an atmosphere enriched in isotopic nitrogen. A significant gain of the isotope occurred with the growth of the following plants: *Coprosma robusta* and *Prunus armeniaca* both showed fixation in leafy shoots; *Pinus radiata*, *Epilobium erectum* and *Dactylis glomerata* showed fixation of nitrogen in the roots. The process may be due to micro-organisms associated with the plants, such as bacteria in stipular glands, or fungi in mycorrhizal roots.

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Some Effects of the Continuous Removal of Floral Buds on the Growth of the Cotton Plant

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With three Figures in the Text

ABSTRACT

Plants of BP52 Upland cotton which were continuously disbudded up to 23 weeks and 35 weeks after planting produced an average of 280 and 764 flower buds per plant respectively. Control plants produced only 112 and 160 flower buds per plant over comparable periods. Disbudded plants produced significantly more flower buds on main-stem sympodia than did control plants; extensive development of monopodial branches with secondary sympodia also contributed to the increased number of buds produced by treated plants. Disbudded plants were taller than control plants at both 23 and 35 weeks. No increase in the dry weight of the aerial parts of control plants occurred between 23 and 35 weeks, although for disbudded plants there was an increase of 25 per cent. over this period. No major qualitative differences in amino-acid, organic acid, or sugar content were observed between treated and control plants at either stage. Between 23 and 35 weeks the absolute amounts of total nitrogen, soluble nitrogen, total sugars (excluding starch and cellulose), and reducing sugar held in stem and leaf material of control plants declined slightly, probably due to diversion of metabolites to developing bolls. Over the same period there was a slight increase in the amount of nitrogen and sugars held in the aerial parts of treated plants.

These results are discussed with reference to the indeterminate fruiting habit of the cotton plant, and to the variable effects of disbudding on yield reported in the literature. The data support the classical theory of cotton plant nutrition in that there is evidence that maturation of bolls proceeds using nutrients which could otherwise be used for further growth and development of fruiting structure.

INTRODUCTION

THE cultivated cotton plant is characterized by an indeterminate growth habit. The main-stem can bear lateral branchs of two types. Frequently, but not always, the lower branches are monopodia, often carrying secondary sympodial branches, whilst the upper branches are usually sympodia. Occasionally monopodial branches may occur side by side with sympodial branches at upper nodes on the main-stem. Because of the indeterminate development of fruiting structure[1] the number of fruiting points produced by different plants growing under similar conditions may vary

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[1] In this paper the term 'fruiting structure' is reserved for that part of the plant framework bearing bolls, buds, and flowers which are referred to as *fruiting bodies*. *Fruiting points* are those points on the fruiting structure which bear, or have borne, fruiting bodies.

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considerably. Furthermore, loss of fruiting bodies due to shedding may be compensated by development of new buds elsewhere on the plant. Both these factors are of considerable economic importance.

A number of authors (e.g. Eaton, 1931 *a*, *b*; Eaton and Ergle, 1952; Singh and Choudri, 1937; Hamner, 1941; &c.) have removed flower buds from plants for various periods in attempts to improve yield. A consistent finding from experiments of this sort is that disbudded plants grow taller and have a higher dry weight than control plants although the effects on yield have varied. The experiment described here represents an investigation into the effects of disbudding on various characters of the cotton plant with particular reference to continued growth and production of new buds. The growth of disbudded plants has been compared with that of control plants allowed to set bolls. Data on the morphological effect of disbudding are presented in Section 1, and results of certain chemical analyses are given in Section 2.

METHODS

Plants of the Upland Cotton variety BP52/8MB were grown at a spacing of 1 ft. on 20 ridged rows 15 ft. long and 3 ft. apart. Sowing date was July 26, 1957 which is about 6 weeks after the optimal planting period (Manning, 1949). Five groups of rows of plants, randomly distributed, comprised the experimental material.

Gp. 1: 4 rows containing 44 plants in all. Control plants sampled 23 weeks after planting (see below).

Gp. 2*a*: 4 rows containing 56 plants in all. All young floral buds with bracts exceeding 5 mm. wide were removed at weekly intervals from weeks 10–23 after planting. Plants sampled at 23 weeks.

Gp. 2*b*: 2 rows containing 27 plants in all. Plants disbudded in the same way as Gp. 2*a* plants, but allowed to develop fruiting bodies between the 23rd and 35th week, and sampled at the 35th week.

Gp. 3: 5 rows containing 64 plants in all. Control plants sampled at the 35th week.

Gp. 4: 5 rows containing 58 plants in all. Plants disbudded from week 10 until week 35, and then sampled.

Records were kept of the numbers of buds removed from each treated plant. At harvest, the following observations were made following Farbrother's method (1958); height of main axis, number of nodes on main-stem bearing monopodia or sympodia, numbers of fruiting bodies of different types, and their position on the plant. Dry weight of stems, petioles, leaves, and fruiting bodies was also measured. Occasional observations were made on fruiting-body production by plants of groups 1 and 3 during the experiment. For this 10 plants were sampled *in situ*.

The experimental plots were irrigated by an overhead sprinkler whenever the moisture tension in the top foot of soil rose to give a value of 3·25 log Ohms as measured by nylon-stainless steel units (Farbrother, 1957).

Chemical Analyses

All analyses were performed on material macerated in a C. and N. laboratory mill after being dried at 70° C. Qualitative studies were performed by paper chromatography using Whatman No. 1 filter paper.

Soluble Nitrogen Compounds. An extract of material in 70% ethanol was concentrated *in vacuo* and chromatographed in two dimensions using the ascending method. The solvents used were saturated phenol-water (3 g. per ml.) and *n*-butanol-acetic acid-water (3 : 1 : 1). Spots were developed with ninhydrin and identified by position, or after elution, by running against known marker compounds.

Sugars. Material was extracted with cold water. The extract was concentrated over a water bath and chromatographed by the descending method using either *n*-butanol-formic acid-water (Wiggins and Williams, 1952) or ethyl acetate-acetic acid-water (3 : 1 : 3) as solvents. Spots were developed with 1 per cent. $KMnO_4$ in 2 per cent. Na_2CO_3 (Pacsu, Mora, and Kent, 1949), and identified by position relative to known marker compounds.

Organic acids. Acidified aqueous extracts of plant material were extracted with peroxide-free ether in a Kutscher-Steudel apparatus for 18 hours. The ether extract was evaporated to dryness, redissolved in a small volume of water and chromatographed by the descending method using ethanol-chloroform-90 per cent. formic acid (65 : 33 : 2) or *iso*-propanol-0·88 ammonia-water (3 : 1 : 1). Spots were developed with 0·04 per cent. bromcresol green and identified by position relative to known marker compounds.

A number of quantitative analyses were also performed.

Nitrogen. For Total Nitrogen plant material was digested with H_2SO_4 in presence of a selenium-sodium carbonate catalyst. Nitrogen in the digested extract was determined by Nesslerization or occasionally by distillation into boric acid (Yuen and Pollard, 1952). For Soluble Nitrogen, material was extracted with 70 per cent. ethanol and this extract was digested and analysed as described above.

Sugars. Material was extracted by intermittent shaking with cold water for 2 hours. The extract was cleared with lead acetate, and excess lead removed with sodium sulphate. Reducing sugars in the cleared extract were determined by a modified Somogyi method (MacDonald and DeKock, 1958). Total sugars were determined after hydrolysis of the extract with dilute H_2SO_4. Starch was occasionally determined. For this, material was extracted with hot water and the resulting extract treated with diastase preparation as described by Eaton and Rigler (1945).

RESULTS

1. Morphological Effects of Disbudding

The production of floral buds. During the early part of the season, the rate of fruiting-point production was similar for all treatments (Fig. 1).

By week 17, production of new floral buds by control plants which were then carrying young bolls showed signs of decline, although disbudded plants continued to produce new buds at a rapid rate up to the end of the experiment. The numbers of fruiting points produced per plant by week 35 were as follows: Gp. 4 (continually disbudded plants) 764, Gp. 3 (control plants) 160, Gp. 2*b* (partially disbudded plants) 345. The data show that there is a decline in the production of new floral buds if plants are allowed to set bolls. Disbudding reduces this effect, but on stopping disbudding the rate of bud production quickly tails off. Thus in the 12-week period after disbudding was stopped, plants of Gp. 2*b* produced only 68 fruiting points per plant. Buds that were not shed flowered quickly giving malformed flowers and small abnormal bolls not exceeding about one-third of the normal size. This is probably an ageing effect. Mr. L. C. Hughes (personal communication) obtained a similar result after prolonged disbudding of cotton in the Sudan.

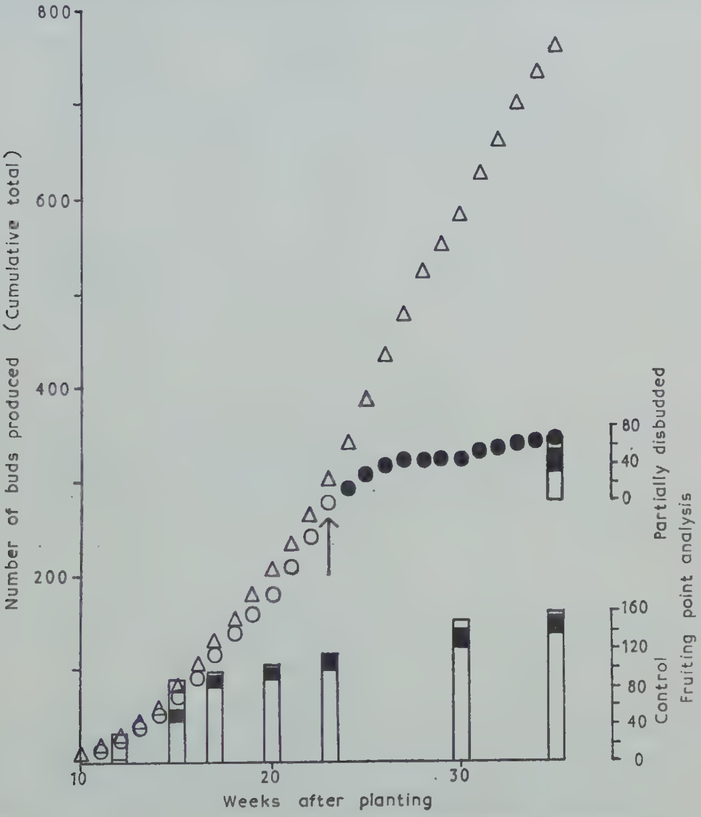

FIG. 1. The total number of buds produced over various periods by plants of Gp. 2*a* (open circles), Gp. 2*b* (closed circles), and Gp. 4 (triangles). For control plants of Gps. 1 and 3 a fruiting-point analysis is shown. The upper open square in the histogram represents numbers of buds, the closed square the number of bolls, and the lower open square the number of fruiting bodies shed. Fruiting-point analysis of plants of Gp. 2*b* is also given.

Effects on plant height, main-stem node number, and internode length. Disbudding had a significant effect in increasing plant height (Fig. 2).

For plants sampled at week 23 this was accounted for entirely by a greater number of nodes in treated plants. Of plants sampled at week 35, those which had been continuously disbudded had a significantly higher mean internode length than either control or partially disbudded plants. Both sets of disbudded plants had a greater number of main-stem nodes than control plants. Between the 23rd and 35th week, plants from Gps. 3 and 2*b* put on 6 and 5 main-stem nodes respectively whilst continuously disbudded plants put on 10 nodes.

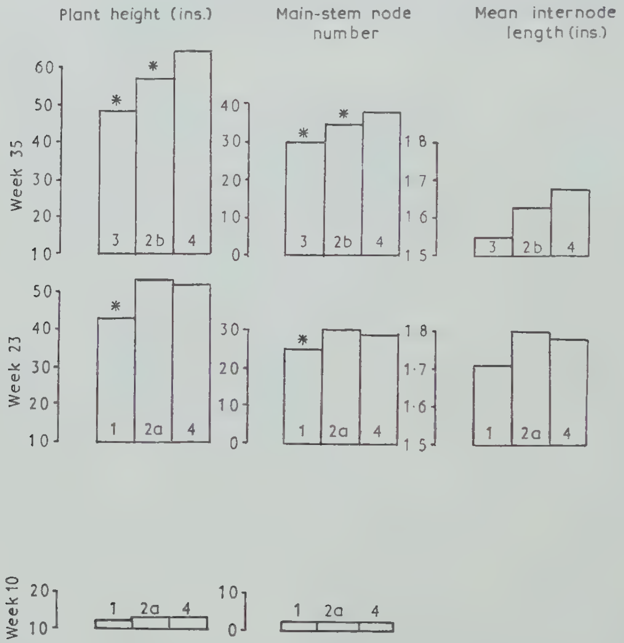

FIG. 2. Height, main-stem node number, and mean internode length for plants of various groups at 10, 23, and 35 weeks. Asterisks indicate significant differences at the 5 per cent. level.

The position of fruiting points. Disbudded plants produced 280 buds per plant in the period up to 23 weeks whereas over the same period control plants produced only 112 buds per plant. The location of the extra fruiting points on the fruiting structure of disbudded plants was investigated.

From Fig. 2 it will be seen that Gp. 2*a* plants carried 5 more nodes on the main stem than control plants. These upper nodes gave rise to sympodial branches carrying at the most 3 flower buds, and could thus account for only 15 of the extra buds.

The mean number of buds borne on all the sympodial branches for 20 plants from each group were as follows:

(*a*) For Gp. 1 plants 3·79 buds per sympodium;
(*b*) For Gp. 2*a* plants 6·12 buds per sympodium.

This difference is highly significant ($t = 4{\cdot}4$, 248 d.f.).

Since control plants carried an average of 20, and treated plants an average of 25 sympodial branches, the number of buds borne on main-stem sympodia is:

(*a*) For control plants 76 fruiting points per plant;
(*b*) For disbudded plants 153 fruiting points per plant.

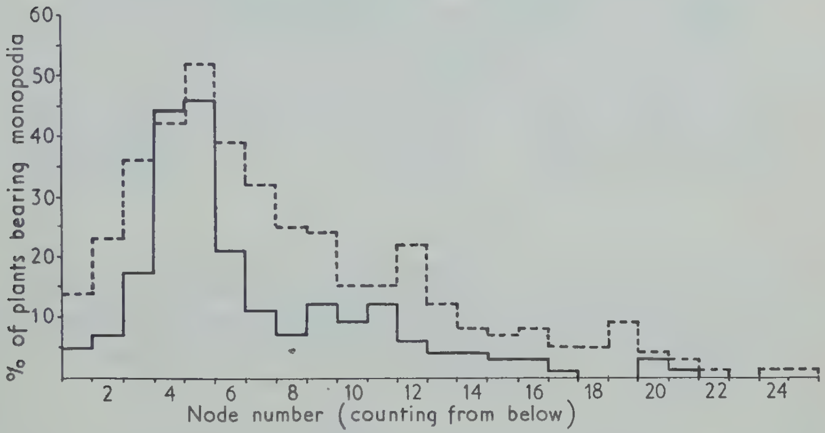

FIG. 3. The percentage of plants bearing monopodial branches at a given node. Broken line represents treated plants and solid line control plants.

Thus the number of fruiting points held on monopodial branches is 36 for control plants, and 127 for disbudded plants. Development of fruiting points from monopodial branches constitutes an important effect of disbudding. In addition, the average numbers of monopodial branches on the main-stem differ significantly between the two groups, being for control plants 4·1 and for treated plants 7·5 ($t = 3{\cdot}3$, 98 d.f.). The location of these monopodial branches on the main-stem is shown in Fig. 3. Analysis of numbers of main-stem nodes bearing both sympodia and monopodia showed that disbudded plants produced twice as many monopodia as control plants. For disbudded plants 64 per cent. of these monopodia bore more than 5 fruiting points whereas the comparable value for control plants was 25 per cent.

That disbudding considerably increases the fruitfulness of all monopodia will be seen from Table 1 which summarizes the relative production of fruiting points by branches of the two types.

Changes in plant dry weight. Eaton (1931*b*) found that disbudding increased the total dry weight of treated plants. In the present experiment roots were not sampled, but the aerial parts of treated plants were found to have a higher dry weight than those of control plants (Table 2). This was largely accounted for by an increase in the amount of stem material as might be expected from the previous results.

Aerial parts of control plants showed no significant increase in dry matter content between weeks 23 and 35, and any increase in dry matter due to carbon assimilation must have been counter-balanced either by losses due to shedding of leaves or buds (leaf dry weight decreased slightly over the period and some bud shedding is shown in Fig. 1) or by transport of assimilates to the root system. The new fruiting points produced over this period (see Fig. 1 and Table 2) were presumably produced at the expense of stored nutrients.

TABLE 1

The Percentage of Fruiting Points borne on Sympodial or Monopodial Branches

Mean numbers of fruiting points per plant are given in parentheses

	Week 23		Week 35	
Branch type	Control	Disbudded	Control	Disbudded
Sympodia .	(76) 69	(153) 55	(88) 55	(304) 40
Monopodia	(36) 31	(127) 45	(72) 45	(456) 60

TABLE 2

The Dry Weight (in grams) of Aerial Portions of Treated and Control Plants together with the Number of Fruiting Points Produced per Unit Weight of Tissue. Standard Error of the Mean is Quoted for Weight Data

	23 weeks	35 weeks
Wt. of aerial parts		
Control . .	142±5·1	150± 7·2
Disbudded . .	254±9·7	318±10·1
Wt. of stem tissue		
Control . .	65±3·2	60± 3·7
Disbudded . .	182±7·5	228± 7·9
Wt. of leaf tissue (at time of sampling)		
Control . .	22±2·9	15± 1·7
Disbudded . .	72±4·2	90± 7·3
Wt. of buds and bolls (by subtraction)		
Control . .	55	75
Disbudded . .	—	—

	23 weeks	35 weeks
F. pts./g. of aerial part		
Control . .	0·79	1·06
Disbudded .	1·10	2·39
F. pts./g. of stem tissue		
Control . .	1·72	2·60
Disbudded .	1·54	3·34

Correlation of fruiting-point number with the amount of material in aerial parts of the plant showed that at both weeks 23 and 35 disbudded plants had produced a greater number of buds per unit weight than control ones. Over this period the number of fruiting points per unit weight of stem doubled for disbudded plants, in spite of the fact that the weight of stem tissue increased by less than 20 per cent.

2. *The Effect of Disbudding on the Chemical Constituents of the Aerial Portions of Plants*

Qualitative analyses. There were no major differences in amino-acids, sugars, and organic-acid constituents of control and disbudded plants either at weeks 23 or 35, and for the sake of continuity these data are presented as an appendix.

Quantitative analyses. The total nitrogen, and sugar content of leaf, stem, and buds of control plants expressed on a percentage basis was found to decline between weeks 23 and 35 (Table 3). Total nitrogen in disbudded plants increased over the period but the percentage of total sugars declined slightly.

TABLE 3

Nitrogen and Sugar Constituents of Control and Disbudded Plants at 23 and 35 Weeks, Expressed as a Percentage of Dry Weight

NITROGEN	Total			Soluble		
	Leaf	Stem	Bud	Leaf	Stem	Bud
23 weeks						
Control plants	4·01	0·96	3·56	0·46	0·25	1·05
Disbudded plants	2·58	0·66	—	0·52	0·31	—
L.S.D. 5%	0·48	0·21	—	0·16	0·08	—
35 weeks						
Control plants	3·44	0·89	2·96	0·40	0·25	0·95
Disbudded plants	2·83	0·81	—	0·57	0·20	—
L.S.D. 5%	0·51	0·17	—	0·12	0·08	—

SUGARS	'Total'			Reducing		
	Leaf	Stem	Bud	Leaf	Stem	Bud
23 weeks						
Control plants	1·45	4·31	—	0·51	0·78	—
Disbudded plants	1·39	2·01	—	0·49	0·51	—
L.S.D. 5%	0·16	0·80	—	0·051	0·17	—
35 weeks						
Control plants	1·12	2·90	—	0·37	0·54	—
Disbudded plants	1·28	1·96	—	0·41	0·56	—
L.S.D. 5%	0·12	0·71	—	0·031	0·10	—

By combining data from Tables 2 and 3 the changes as grams per plant can be derived and these present a rather different picture (Table 4).

For control plants the total amounts of nitrogen and sugars fall with time for both stem and leaf material. The decrease in sugars is somewhat greater than that for nitrogen. These losses may be due to a number of factors: (*a*) diversion of materials to developing bolls, (*b*) losses due to respiration, or from shedding of leaves, buds, or young bolls, or (*c*) diversion of materials to the root system. Probably (*a*) is the most important factor; unpublished data on lint formation over this period show that this can account for utilization of up to 2 g. of carbohydrate per plant. Developing seeds would require considerable amounts of nitrogen. If (*a*) is accepted as a likely explanation, it follows that what have been considered as 'losses' are not losses at all, but an indication of a redistribution of stored materials. Lack of data for boll and root material makes confirmation of this idea impossible.

TABLE 4

The Nitrogen, Sugar, and Starch Content of Stem and Leaf Material from Control and Disbudded Plants, Expressed as Grams per Plant

	Total N	Soluble N	'Total' sugars	Reducing sugars	Starch
CONTROL PLANTS					
Stem					
Week 23 .	0·624	0·613	2·80	0·507	2·80
Week 35 .	0·534	0·150	1·74	0·324	2·92
Change .	−0·090	−0·013	−1·06	−0·183	+0·12
Leaf					
Week 23 .	0·882	0·101	0·319	0·1122	
Week 35 .	0·516	0·060	0·168	0·0555	
Change .	−0·366	−0·051	−0·151	−0·0567	
DISBUDDED PLANTS					
Stem					
Week 23 .	1·20	0·56	3·66	0·93	9·30
Week 35 .	1·85	0·46	4·47	1·28	9·27
Change .	+0·65	−0·10	+0·81	+0·35	−0·03
Leaf					
Week 23 .	1·86	0·37	1·001	0·353	
Week 35 .	2·54	0·51	1·115	0·369	
Change .	+0·68	+0·14	+0·114	+0·016	

For disbudded plants there is an increase in the nitrogen and sugar content of the aerial portions with time. The increased nitrogen content can be explained either by a continued uptake of nitrate by the roots, or else by a movement of stored nitrogen from the roots to the aerial parts of the plants. Between 23 and 35 weeks leaf weight per plant rose from 72 g. to 90 g. and since the percentage of sugars in stem and leaves remains constant with time it would seem that there is either appreciable synthesis of new sugars or a movement of carbohydrate from the roots to the aerial parts.

DISCUSSION

The Effect of Disbudding on Yield

Experiments designed to investigate the effects of disbudding on yield of seed cotton have given variable results. For instance, in the United States, Hamner (1941) removed all flower buds for a 6-week period and found this to have no effect on yield, whilst Dunnam, Clark, and Calhoun (1943) found disbudding for periods of from 4 up to 9 weeks reduced yield, although disbudding for up to 4 weeks had no effect. Eaton (1931*a*) found that removal of all flowers for the first 24 days of the flowering period substantially increased final yield. At Ilonga, in Tanganyika, McKinlay and Geering (1957) found that disbudding for up to 12 weeks had no effect on yield. The period for which disbudding can be practised without reducing yield is apparently dependent upon the length of time during which growth is not seriously impaired by environmental factors (e.g. water strain, low temperatures, &c.). In other words, disbudding does not reduce yield if environmental factors subsequent to the disbudding period allow continued growth, flowering, and boll setting. This hypothesis is supported by data of McKinlay and Geering (1957) and Coaker (1957) who showed that, at Namulonge, loss of fruiting bodies due to insect attack does not necessarily reduce the yield of early planted cotton, for, provided plants do not suffer excessively from water strain, growth late in the season enables new bolls to set and compensate for the early season loss.

With the late-planted cotton in this experiment it was thought that continued growth of partially disbudded plants (Gp. 2*b*) into the dry season (January–February) would occur since the soil moisture content was kept high by irrigation. In fact disbudding for 13 weeks had an adverse effect on yield, for although more bolls were set on treated plants than on controls, these were small and of little commercial value. Control plants set a few normal bolls over the same period (i.e. 23–35 weeks) suggesting that soil moisture was probably not limiting and that the effect was not a seasonal one. In addition to the environmental factors determining the response of plants to the discontinuation of disbudding, there is apparently a further factor, inherent in the plant, leading to the production of small and malformed bolls. The interaction of these factors will determine the effect of disbudding on yield.

Data in Table 1 show clearly that increase in fruiting structure as a result of disbudding comes from further growth of both sympodial and monopodial branches. These data were obtained for a cotton variety generally bearing two or three large monopodial branches at the lower main-stem nodes (Fig. 3). Balls (1915, Pl. X, p. 147) shows a photograph of what is described as 'the ideal cotton plant'. This has no monopodial branches, and growth of the main-stem ceases soon after fruiting commences. How does such a plant respond to disbudding? Direct evidence is not available but the variety U4, which has poorly developed monopodial branches (see photographs in Parnell, 1926, 1927), when grown under Uganda conditions suffers heavily from shedding due to insect pests and does not show continued compensatory growth and thus yields lower than BP52 selections (see Namulonge Progress Reports after 1949–51). While this phenomenon may be a response to an adverse environment (which is not, it follows, adverse for growth of BP52) it may be linked with an inherent inability of such cotton to continue growth and production of new fruiting points indefinitely at a rate sufficiently fast to enable a crop to be set within the normal cotton season. The effect of the indeterminate growth habit on yield in such cotton is masked by a slow growth-rate in the latter part of the growing season. Heath's data (1956) support this and are discussed below.

Disbudding and Continued Growth

Mason (1922) claimed that the cessation of growth of the main axis of Sea Island Cotton observed by Harland (1918), and increased susceptibility to shedding could be correlated with a factor tending to channel metabolites from the plant apex to the basal fruiting branches. This hypothesis forms the basis of the nutritional theory of bud and boll shedding. The present results can be considered in relation to the nutrition of fruiting cotton plants as discussed by Mason (1922), Mason and Phillis (1934), Crowther (1934), and Eaton and Ergle (1952).

Increased growth of individual sympodial branches, production of more and bigger secondary sympodia from buds which would normally remain dormant, are all responses of plants to disbudding and it may be concluded from their vigorous development that these stem and branch apices are well supplied with necessary nutrients. The increase in dry weight of treated plants during the latter part of the experiment supports this. Eaton (1931*b*) found that increased development of the root system was characteristic of disbudded plants. The increase in nitrogen in the aerial parts of treated plants is indirect evidence of the continued function of the root system in the present experiment. The net increase in carbohydrate content of aerial parts almost certainly indicates continued carbon assimilation, and it is probable, by analogy with the work of Eaton (1931*b*) and Eaton and Rigler (1945), that movement of carbohydrate to the root system occurs over the latter part of the experiment.

In control plants development of vegetative apices was far more limited, and this is connected with the presence of fruiting bodies on the plant. On Mason's hypothesis, when fruiting commences some kind of controlling mechanism is either brought into play or modified, to effect partition of available nutrients between potential 'sinks'. Such a mechanism probably allows, as Crowther (1934) and others have suggested, diversion of nutrients to developing bolls rather than to roots or branch apices. As a result, few leaves are formed and carbon assimilation declines. Similarly the development of new roots may be reduced, leading to a decline in uptake of nitrogen. Inevitably growth must decline still further until the stage is reached, as between 23 and 35 weeks (Table 2), that growth as measured by increase in dry weight has virtually ceased. Farbrother (1958) found that, at Namulonge, the development of fruiting structure could be adversely affected by water strain, and for plants carrying maturing bolls this effect could be manifested by an almost complete cessation of bud production. This finding is explicable on the nutritional hypothesis advanced if it is assumed that water stress is accompanied by a lowered rate of photo-synthesis (unpublished data on stomatal behaviour support this), and a decrease in uptake of nutrients by the roots.

The nature of the mechanism controlling the partition of nutrients is unknown. Heath (1956) described an experiment at Barberton, S. Africa, in which it was found that a decline in the growth rate of the main axis of normal and disbudded cotton (var. U4) occurred simultaneously when the control plants reached the stage of 'first flower'. Heath suggested that this phenomenon was associated with changes, possibly hormonal, in the apical meristem. Mason (1922) pointed out, however, that when stem apices from plants whose main axis had ceased growth were budded on to young plants, there was an immediate resumption of rapid and vigorous growth from the apparently 'senescent' terminal bud. Data from the present investigation show that the presence of fruiting bodies is important in causing a reduction in growth and it may be that the primary site of the mechanism is to be found in developing fruit. If this is so, it must be admitted that the decline in growth-rate of the main axis of Heath's disbudded plants remains inexplicable, although it is in accord with the suggestion already made, that growth following extensive loss of fruiting bodies occurs at a low rate for cotton of the U4 type.

The rapid decline in growth-rate of the main axis of control plants in Heath's experiment (i.e. from 2 cm. per day to zero, over a 5-week period) stands in contrast to our finding that growth of the main axis of control plants continues up to 35 weeks. Both plant height and main-stem node number increased, in spite of the fact that the dry weight of stem tissue remained constant. This can only be explained by a redistribution of nutrients within the plant framework. Clearly not all available metabolites were used in maturing the bolls set. It may be that in the U4 type cottons, with few vegetative branches, there is no reserve of available nutrients to enable continued growth of the main axis to occur.

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APPENDIX

Points of interest in the chromatographic analyses given below are:

1. Of the amino-acids, alanine and glutamine were frequently present in high concentration in all the tissues sampled. Aspartic and glutamic acids were also present in all tissues although in variable concentration. Tissues yielding glycine gave large amounts of this acid at week 23 but far smaller amounts when sampled at week 35. Tentative comparison of R Alanine

Qualitative Chromatographic Analysis of Chemical Constituents of Control and Disbudded Plants, at 23 and 35 Weeks*

	23 weeks					35 weeks							
	Gp. 1			Gp. 2*a*		Gp. 3			Gp. 2*b*			Gp. 4	
	Leaf	Stem	Buds	Leaf	Stem	Leaf	Stem	Buds	Leaf	Stem	Buds	Leaf	Stem
AMINO ACIDS													
Alanine	+	+	+	+	+	+	+	+	+	+	+	+	+
Asparagine	+	+	+	+		+	+	+	+	+	+	+	+
Aspartic	+	+	+	+	+	+	+	+	+	+	+	+	+
Glutamic	+	+	+	+	+	+	+	+	+	+	+	+	+
Glutamine	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycine	+		+	+		+		+	+		+	+	
Phenyl Alanine	+	+		+		+	+		+			+	
Serine	+												
Threonine	+					+							
Valine	+			+		+			+			+	
Un A†	+			+									
B†	+			+		+			+			+	
C†		+			+		+			+			+
SUGARS													
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+			+									
CARBOXYLIC ACIDS													
Oxalic		+			+	+	+		+	+		+	+
Malic	+	+	+	+	+	+	+	+	+	+	+	+	+
Citric	+		+	+			+	+	+		+	+	
Succinic			+										
Fumaric			+					+			+		
Un 1‡	+	+		+		+	+					+	+

* For leaf and stem material 10 plants were sampled for each group and (+) indicates that the substance was present in at least 6 of the 10 chromatograms. For bud material only 3 plants were sampled and (+) indicates presence of the spot in at least 2 out of 3 chromatograms. Spots occurring with less than these frequencies are not reported.

† R Alanine values for unknown spots

Spot	A	B	C
phenol/water	0·74	0·81	1·25
n-butanol/acetic acid/water	1·11	0·68	1·08
Ninhydrin reaction	Purple	Purple	Purple

‡ R_F value for unknown spot 1

ethanol/chloroform/formic acid	0·56
iso-propanol/NH_3/H_2O	0·28

values for the 3 unknown spots with Fowden and Steward's (1957) published values for unknown compounds in the Liliaceae suggests that Spot A may be equivalent to their Unknown 133, Spot B to Unknown 124, and Spot C to Unknowns 107 or 138. Only small amounts of these compounds were extracted and a more definite identification is not possible.

2. The major carbohydrate constituent identified was sucrose, and this is in agreement with quantitative studies (see Table 3).

3. Of the organic acids, malic was found in considerable amounts in all the material investigated. There was an apparent increase in the amount of oxalic acid present in tissues sampled after 35 weeks.

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